Master of Pharmacy Thesis

Synthesis and evaluation of new gadolinium based MRI contrast agents

Tormod Fjerdrumsmoen

Department of Medical Chemistry
Faculty of Mathematics and Natural Sciences
University of Oslo
2004

ACKNOWLEDGEMENTS

This work has been carried out at the Department of Medical Chemistry, School of Pharmacy, University of Oslo during the period November 2003 to November 2004. My supervisors have been Professor Jo Klaveness and Associate Professor Pål Rongved.

I would like to thank Jo Klaveness and Pål Rongved for the opportunity to carry out this study and for everything they have contributed with.

I am most grateful to my colleague Hong Diem Thi Nguyen for collaboration and social atmosphere.

I would also like to thank Iuliana Johansen for support and valuable assistance.

At last I would like to thank my girlfriend Ranveig, family and friends for encouragement and support during the year.

Tormod Fjerdrumsmoen

Oslo, November 2004

CONTENTS

1 ABSTRACT

In this work three new macrocyclic gadolinium complexes (Gd-DO3A-ADA, Gd-DO3A-MAD and Gd-DO3A-APP) have been syntesised. This was done in order to study how the introduction of hydrophobic groups on DO3A affected the albumin binding and hence the T_1-relaxivity. Gd-DTPA was used as a control in the study.

Gd-DO3A-MAD showed the highest degree of albumin binding (60%). Gd-DO3A-ADA did also have some affinity for albumin (19%) while Gd-DO3A-APP did not have any degree of albumin binding at all.

The T_1-relaxivity for all substances was close to 4.0mM^{-1} s^{-1} in a phosphate buffer solution (pH 7.4). In a 4% BSA solution the T_1-relaxivity increased for Gd-DO3A-MAD and Gd-DO3A-ADA. Gd-DO3A-APP did not show any significant increase in the T_1-relaxivity.

It was attempt to displace Gd-DO3A-MAD from albumin by adding oxazepam, warfarin and caprylic acid to the solution. By adding caprylic acid to albumin before Gd-DO3A-MAD, a decrease in the T_1-relaxivity was observed compared to the solution of only Gd-DO3A-MAD in albumin.

2 ABBREVIATIONS AND NOMENCLATURE

2.1 Abbreviations

δ	Chemical shift in ppm
bs	Broad singlet
BSA	Bovine serum albumin
Cyclen	1,4,7,10-tetraazacyclododecane
d	Dublett
DO3A	1,4,7-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecan
DTPA	Diethylentriaminepentaacetic acid
EI	Electroionisation
ES	Electrospray
Gd-DO3A-ADA	Gadolinium-1,4,7-Tris (carboxymethyl)- 10-[12-(2-acetamido) dodecanoic acid]- 1,4,7,10-tetraazacyclododecan
Gd-DO3A-APP	Gadolinium 1,4,7-Tris (carboxymethyl)- 10-[(L)-2-(2-acetamido)-3-phenyl-propanoic acid] - 1,4,7,10-tetraazacyclododecan
Gd-DO3A-MAD	Gadolinium 1,4,7-Tris (carboxymethyl)- 10-[methyl 12-(2-acetamido) dodecanoate] - 1,4,7,10-tetraazacyclododecan
Gd-DO3A-MAH	Gadolinium-1,4,7-Tris (carboxymethyl)- 10-[metyl-6(2-acetoxy) hexanoate]- 1,4,7,10-tetraaza-cyclododecan
Gd-DO3A-MAP	Gadolinium 1,4,7-Tris (carboxymethyl)- 10-[(L)-methyl 2-(2-acetamido)-3-phenylpropanoate] - 1,4,7,10-tetraazacyclododecan
HPLC	High Performance Liquid Chromatography
Hz	Hertz
M	Molecule ion
m	Multiplett
Me	Methyl
MRI	Magnetic resonance imaging
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
ppm	Parts per million

R_1	Longitudal relaxations rate
r_1	Longitudal relaxivity
s	Singlett
t	Triplett
T_1	Longitudinal relaxation time
T_2	Transversal relaxation time
Tert-butyl	1,1-dimetylethyl
TFA	Triflouroacetic acid
THF	Tetrahydrofuranone
TLC	Thin layer chromatography
q	Quartet

2.2 Nomenclature

The compounds are given their English names after the IUPAC-system for nomenclature.

3 INTRODUCTION

3.1 Magnetic resonans imaging (MRI)

3.1.1 Introduction

In 1946, Felix Bloch and Edward Purcell independently discovered that the nuclei of different atoms absorbed radio waves at different frequencies[1, 2]. In 1952, Bloch and Purcell received the Nobel Prize for their discovery of what was referred to as Nuclear Magnetic Resonance (NMR), eventually to be known as Magnetic Resonance Imaging (MRI)[3]. In 1970, the medical imaging world significantly changed with the contributions of Dr. Raymond Damidian. Dr. Damidian discovered that the structure and abundance of water in the human body was the key to MR imaging. He showed that tumors' NMR signals differed from those of normal tissue. It was Paul Lauterbur, however, who implemented the concept of tri-plane gradients used for exciting selective areas of the body. In 1975 Peter Mansfield produced the first human NMR image[4, 5].

3.1.2 The basic principles of MRI

Different tissues in the body have different density of hydrogen. MRI is, as mentioned, based on these hydrogen atoms. The protons in these atoms are constantly spinning around and because of that, creating a magnetic field (Figure 3.1).

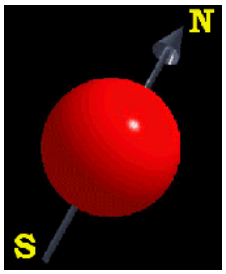
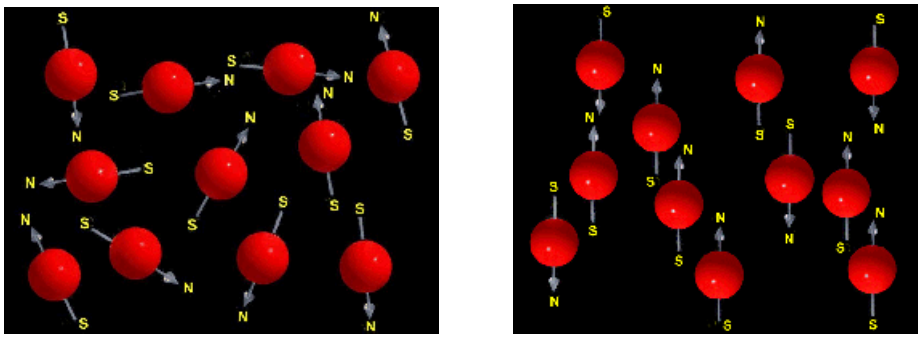


Figure 3.1: Proton spinning and creating a magnetic field

Hydrogen nuclei magnetic moments are randomly oriented in the absence of an external magnetic field and are considered to have a net magnetization of zero. Once hydrogen protons

are placed in the presence of an external magnetic field, they align themselves parallel or anti-parallel to the magnetic field. As more protons tend to aligned themselves parallel than anti-parallel they do not cancel each other out (Figure 3.2).



No magnetic field External magnetic field

Figure 3.2: Protons in magnetic field and in no magnetic field

Hydrogen atoms do not actually align directly with the direction of the magnetic field, but rather rotate around the axis of the magnetic field. This is called precession. The frequency of the precession is called the Larmor frequency and is important because it is the frequency at which the nucleus will absorb energy. The absorption of energy will cause the proton to alter its alignment. The precession frequency is dependent of the magnetic field and is described as follow:

$$W_o = \gamma \times B_o$$

W_o is the precession frequency, γ is the gyromagnetic ratio (of hydrogen in MRI) and B_o is the strength of the magnetic field.

If a radio wave pulse at the Larmor frequency is applied to the nucleus of a hydrogen atom, the protons will alter their alignment from the direction of the main magnetic field to the direction opposite the main magnetic field. The amount of applied energy will decide the deflection of the magnetization. When a 90° pulse is applied the protons will be flipped into the transversal plane.

After the external radio frequency signal is turned off, the system returns to equilibrium by a process called relaxation. Two phenomenon's simultaneously occur.

- Longitudinal magnetization gradually increases and is called T_1 recovery
- Transverse magnetization gradually decreases and is called T_2 decay

T_1 recovery (also called spin-lattice relaxation) is described as the time it takes for 63% of the longitudinal magnetization to reappear, and T_2 decay (also called spin-spin relaxation) is described as the time it takes before 63% of the transverse magnetic plane has disappeared.

3.2 MRI contrast agents

3.2.1 Introduction

The aim of using contrast agents in MRI is their affect on the relaxation times of the protons in the surrounding tissue. This was first published by Lauterbur, Mendoca-Dias and Rudin in 1978[6]. They demonstrated that paramagnetic substances were effective enough that it could be visualized in MRI. The most abundant paramagnetic substances used today are Mn(II), Gd(III) and Fe(III). They all have high magnetic moments. Gd(III) is most common used in nowadays contrast agents because it has some favorable properties (see section 3.2.2). The Gd(III) ion cannot be used as a contrast agent itself because it is toxic. Therefore stabile complexes of the ion have to be prepared. Today 30-40% of all MRI investigations make use of a contrast agent[7].

3.2.2 Gadolinium based contrast agents

Contrast agents based on gadolinium belongs to the group of positive contrast agents. That means they reduce the protons T_1-relaxation time and because of that increase the signal intensity in MRI. Gd(III) is the most used paramagnetic ion in MRI. The most important reason is that Gd(III) has seven unpaired electrons, more than any other ion. Gd(III) also has a symmetric S-state that will give a low electronic relaxation rate[8].

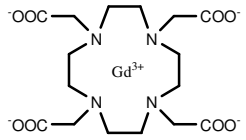
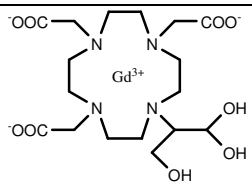
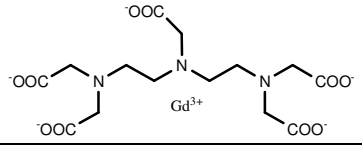
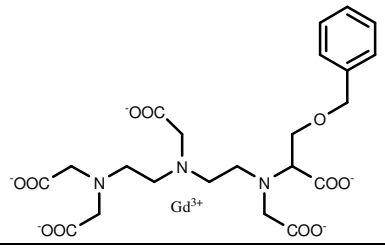
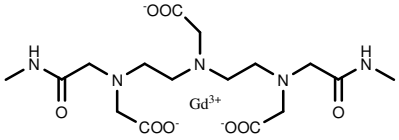
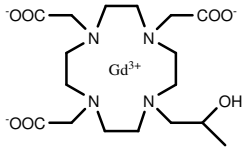
As earlier mentioned Gd(III) is a toxic heavy metal and therefore complexes have to be prepared. This complexes need to have a high stability to avoid *in vivo* dissociation and they have to be biocompatible. Good solubility in water and low osmolality are also preferred properties because the agents are used in quite large quantities.

All current Gd(III) based MRI contrast agents on the European marked are based on either linear or macrocyclic poly(aminocarboxylat)-complexes.

3.2.3 Commercially available Gd(III) based MRI contrast agents

There are currently six Gd(III) based MRI contrast agents on the Norwegian market.

Table 3.1: Commercially available contrast agents on the Norwegian market.

Product name	Contrast agent	Structure
Dotarem®	Gd-DOTA Gadolinium-1,4,7,10-tetra-acetic acid-1,4,7,10-tetraazacyclo-dodecane	
Gadovist®	Gd-DO3A-butrol Gadolinium-10-((hydroxymethyl)-2,3-dihydroxypropyl)-1,4,7-triacetic acid-1,4,7,10-tetreaza-cyclododecane	
Magnevist®	Gd-DTPA Gadolinium-N,N´,N´,N´´,N´´-diethylentriaminepentaacetic acid	
MultiHance®	Gd-BOPTA Gadolinium-(4RS)-(4-carboxy-5,8,11-tris(carboxymethyl)-1-phenyl-2-oxa-5,8,11-triazatridecan-13-oic acid)	
Omniscan®	Gd-DTPA-BMA Gadolinium-N,N´´-bis(methyl-carbamoylmethyl)-N,N´,N´´-tris-diethylentriamine-pentaacetic acid	
Prohance®	Gd-HP-DO3A Gadolinium-10-(2-hydroxypropyl)-1,4,7-triacetic acid-1,4,7,10-tetra-azacyclododecane	

Dotarem®, Gadovist®, Magnevist®, Omniscan® and Prohance® all have relatively similar properties. They are extracellular agents and they are excreted by glomerular filtration. They do not cross the intact blood-brain barrier and this make them well suited for diagnosis of pathologies in the brain. The T_1-relaxivity of the contrast agents is relatively low. This is due to

the low τ_R values and the complexes which can only bind one water molecule (The hydration number, q=1).

MultiHance® is the only gadolinium based MRI contrast agent indicated for imaging of the liver. Because of its anionic and lipophilic properties it is taken up into the hepatocytes by the bilirubin anion system. The lipophilic group of MultiHance® has some affinity for albumin[9]. The bound part of the contrast agent will of that reason have increased τ_R and again increased relaxivity.

Table 3.2: Properties of gadolinium MRI contrast agents on the Norwegian market.

Contrast agent	**Stability Log K**	**Relaxivity ($mM^{-1}s^{-1}$)** (25°C, 20MHz)	**Hydration number (q)**	**τ_R (ps)**	**τ_m (ns)**
Dotarem®	25.3[8]	4.2[8]	1[8]	77[8]	108[8]
Gadovist®	21.8[10]	3.6[11]	NA	NA	NA
Magnevist®	22.5[8]	4.3[8]	1[8]	58[8]	130[8]
MultiHance®	22.6[8]	5.2[8]	1[8]	88[8]	NA
Omniscan®	16.9[8]	4.4[8]	1[8]	66[8]	1000[8]
Prohance®	23.8[8]	3.7(40°C)[8]	NA	57	NA

3.2.5 New generation of MRI contrast agents

The first gadolinium based MRI contrast agents introduced on the marked were unspecific in their distribution. The latest MRI contrast agent approved for the Norwegian marked is MultiHance®. As mentioned, this agent accumulates in the liver and is for that reason an organospecific contrast agent. Currently the phase III clinical trials are completed for another contrast agent, called AngioMARK® (MS-325) (Figure 3.3). This agent binds reversible to albumin and is well documented as a blood pool contrast agent.

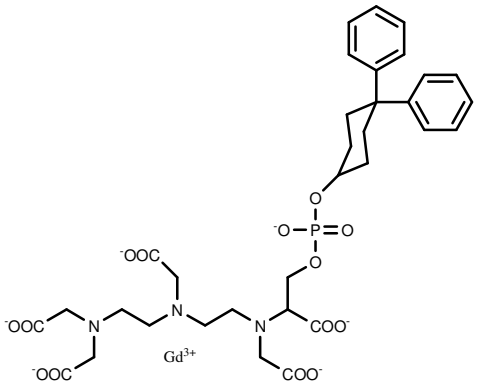


Figure 3.3; AngioMARK®(MS-325)

Lately, the research has begun focus on responsive MRI contrast agents. This means agents that alter their relaxivity as a function of alterations in the microenvironment whereby they distribute. This could be parameters such as pH, pO_2, temperature or enzymatic activity[12, 13, 14, 15].

The trend in the development of new gadolinium based contrast agents can consequently be divided into two directions. The one is targeted compounds. This means contrast agents that will accumulate in a specific organ or area of the body. Especially contrast agents for the blood pool is well investigated. The second is responsive agents. Those are agents that alter their relaxivity because of the microenvironment whereby they distribute. The development of this class of contrast agents have not come as far as the targeted contrast agents, but it is expected that this field will continue to evolve.

At last there are contrast agents that combine the two directions. This means contrast agents that are targeted towards an organ or area of the body and because of this increase their relaxivity. MS-325 is a compound in this class. It will because of binding upon albumin stay in the blood pool. This binding also makes it increase its relaxivity due to increased rotational correlation time.

3.3 Relaxivity

MRI contrast agents are not directly visualized in the MR image. It is the effect of the complex on the nearby proton which is seen. The efficacy of a Gd(III) contrast agent is described by the T_1-relaxivity (r_1):

$$r_1 = \frac{R_1^{obs} - R_1^{m}}{[Gd(\mathrm{III})]}$$

Where R_1^{obs} is the observed relaxation rate, R_1^{m} is the relaxation rate of the matrix and [Gd(III)] is the concentration of Gd(III) in mM. The T_1-relaxation rate of hydrogen atoms in water containing gadolinium could be described from a model considering contribution from water molecules in the inner sphere, (water molecules coordinated to gadolinium, R_1^{is}), outer sphere (bulk water, R_1^{os}), second sphere (water attached to the ligand, R_1^{2s}) and the diamagnetic contribution from the matrix, R_1^{m} .

$$R_1^{obs} = R_1^{is} + R_1^{os} + R_1^{2s} + R_1^{m}$$

When one or more water molecules are coordinated to the gadolinium ion, R_1^{is} is the most important contribution to the T_1-relaxation rate. R_1^{is} can be expressed as:

$$R_1^{is} = \frac{Cq}{55{,}6}\left(\frac{1}{T_{1M} + \tau_M}\right)$$

where C is the molar concentration of Gd(III), q is the number of water molecule(s) coordinated to gadolinium, T_{1M} is the longitudinal relaxation rate of the protons coordinated to gadolinium and τ_M is the time the water molecule(s) is coordinated to gadolinium. The value of T_{1M} is in turn given by the Solomon–Bloembergen-Morgan equation:

$$\frac{1}{T_{1M}} = \frac{2}{15}\frac{2\gamma_H^2 g^2 S(S+1)\beta^2}{r_H^6}\left[\frac{3\tau_{C1}}{1+\omega_H^2\tau_{C1}^2} + \frac{7\tau_{C2}}{1+\omega_S^2\tau_{C2}^2}\right]$$

where γ_H is the proton gyromagnetic ratio, g is the electron g-factor, S is the electron spin quantum number, ß is the Bohr magneton, r_H is the distance between the Gd(III) ion and the inner sphere water protons, ω_H and ω_S are the proton and electron Larmor frequencies and τ_{ci} (i= 1,2) are the correlation times related to the modulation of the electron-proton dipolar coupling. τ_{ci} is a based on three factors:

$$\frac{1}{\tau_{Ci}} = \frac{1}{\tau_R} + \frac{1}{\tau_M} + \frac{1}{\tau_S}$$

where τ_R is the reorientation of the paramagnetic species, τ_M is the residence lifetime of the inner-sphere water molecule and τ_S is the electronic relaxation time to the Gd(III) ion.

From this discussion it is seen that some parameters can be altered to affect the relaxivity. Increasing the hydrating number, increasing τ_R or decreasing τ_M will all involve increased relaxivity.

Increasing the hydration number is often accompanied by a decrease in thermodynamic stability and kinetic inertness. This consequence is undesirable in a contrast agent. Increasing τ_R has been a more successful way to increase the relaxivity. The preparation of macromolecules with covalent linked Gd(III) complexes, Gd(III) complexes which binds non covalent to endogenous macromolecules and multimeric Gd(III) chelates has been successfully in this way[8].

4 AIM OF THE STUDY

The aim of this study has been to synthesise DO3A-derivates with a high degree of albumin binding. Such substances have the potential as high relaxivity, blood pool agents. A contrast agent in this category is already designed and well documented, the previously mentioned MS-325. MS-325 will, because of its high albumin binding, stay in the blood pool and do not diffuse into the extra cellular matrix in any degree. Because of that it will also has a much higher clearance time than the commercially available contrast agents. This will in time mean the substance is more exposed for degradation and therefore the complex has to be very stabile. From the literature it is known that macrocyclic complexes tend to be more stabile than linear complexes[8, 16]. The macrocyclic complexes have a much slower kinetic than the linear complexes and this makes the macrocyclic complexes exceptionally inert and thermo-dynamically stable[8].

Another advantage of the DO3A derivates synthesized in this study is a more simple synthesis than MS-325. While MS-325 is a six step synthesis, the DO3A derivates synthesised here is a 3-5 step synthesis[17].

5 RESULTS AND DISCUSSION

5.1 Strategies and synthesis of new MRI contrast agents

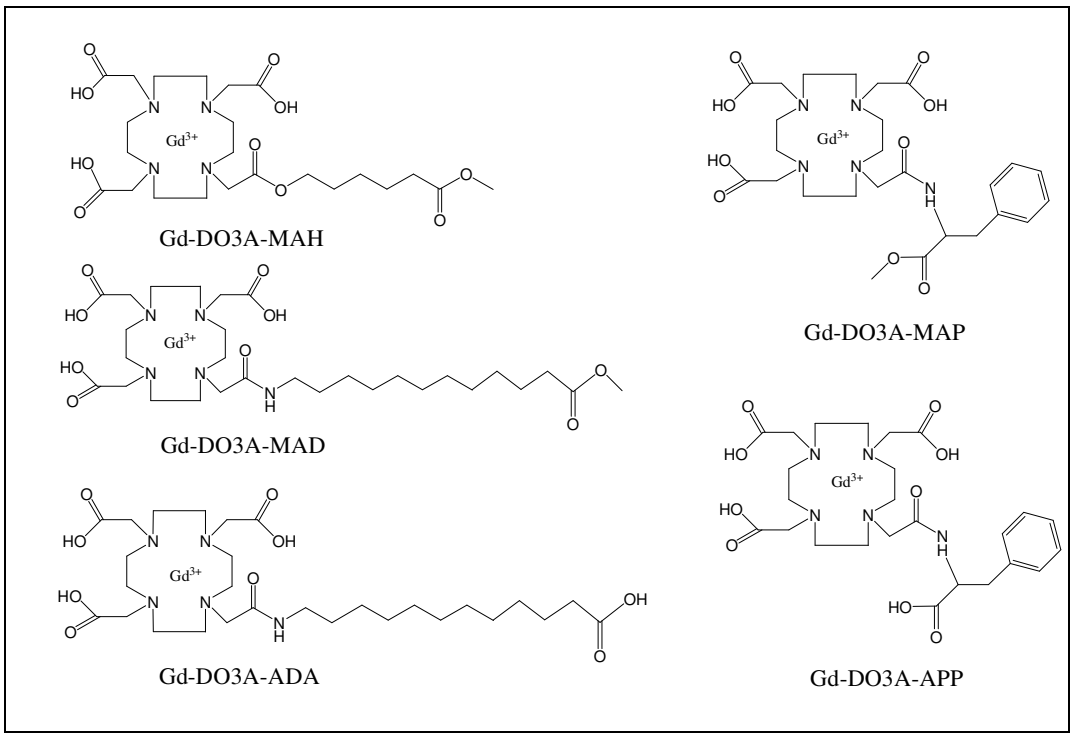


Figure 5.1: Gd(III) complexes attempt to produce

The aim of the study was to synthesise gadolinium-DO3A derivates with high degree of albumin binding. In this attempt different molecules with an assumed high affinity for albumin were coupled to DO3A. From the literature it is known that fatty acids have this quality[18]. Phenylalanine, like the fatty acids, also has a hydrophobic group and a hydrophilic part with a negative charge. NSAIDs and MS-325 which all have high protein binding, share these properties[16, 19]. It could therefore be expected that phenylalanine would have high affinity for albumin. It is known that a wide range of hydrofobic substances have high affinity for albumin and therefore the methyl esters of the acids were synthesised.

The main strategy in the synthesis was to connect these substances with the secondary amine in DO3A (or DO3A-tert-butyl), using chloroacetyl chloride as a connecting link.

5.1.1 Synthesis of DO3A and DO3A-tert-butyl

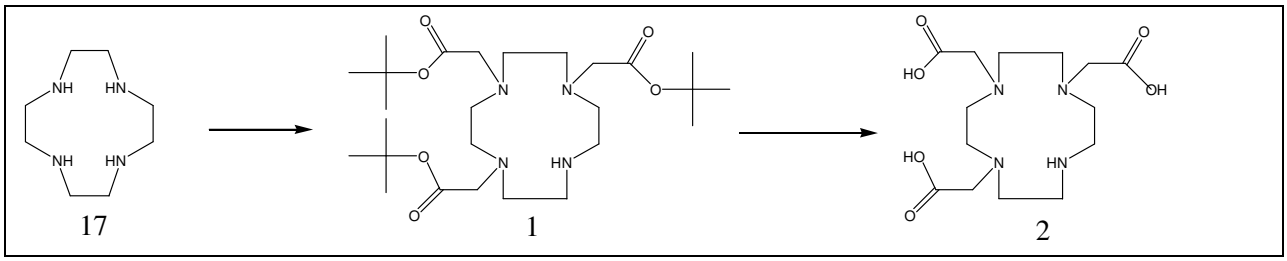


Figure 5.2: Synthesis of DO3A and DO3A-tert-butyl

Synthesis of* 1*,4,7-tris(carboxymethyl-tert-butylester)-1,4,7,10-tetraazacyclodecane (DO3A-tert-butyl)(1)

The synthesis of DO3A-tert-butyl was carried out according to the litterature[20]. The yield (40%) was a little lower than expected. ^{13}C-NMR and MS(ES$^+$) confirmed the reaction had taken place and indicated a pure product.

Synthesis of 1,4,7-tris(carboxymethyl)-1,4,7,10-tetraazacyclodecane (DO3A)(2)

The tert-butyl groups of DO3A-tert-butyl had to be removed to give DO3A. This was carried out using TFA. TFA is often used for the cleavage of tert-butyl esters[21]. The yield of this reaction was 96%. ^{13}C-NMR and MS(ES$^+$) confirmed the reaction had taken place and indicated a pure product.

5.1.2 Strategy for synthesis of Gd-DO3A-MAH (Gadolinium-1,4,7-tris (carboxymethyl)- 10-[metyl-6(2-acetoxy)hexanoate]- 1,4,7,10 -tetraazacyclododecan)

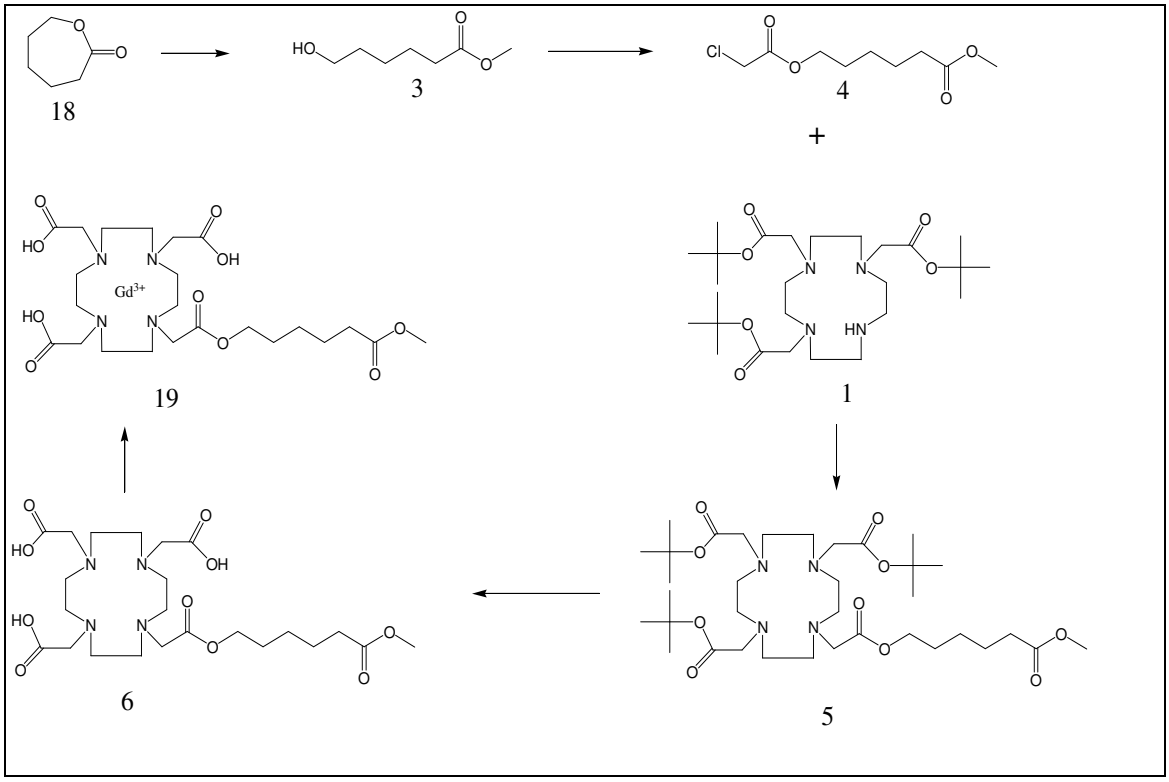


Figure 5.3: Strategy for synthesis of Gd-DO3A-MAH

The synthesis strategy for DO3A-metyl-6(2-acetoxy)hexanoate was as described in figure 5.3. The reaction of DO3A-tert-butyl with alkyl halides is earlier described in the literature[23].

Synthesis of methyl-6-hydroxyhexanoate (3)

The ε-caprolactone was reacted with methanol using concentrated sulfuric acid as a catalyst. After evaporation of the solvent, the oil was solved in water and neutralized by the addition of $NaHCO_3$. There was no phase separation so the water was extracted with diethylether. After evaporation, TLC showed the substance was not pure so the oil was vacuum distilled. ^{1}H-NMR, ^{13}C-NMR and MS(EI) confirmed the substance was synthesised.

Synthesis of methyl 6-(2-chloroacetoxy)hexanoate (4)

The reaction between chloroacetyl chloride and primary alcohols is earlier described in the literature[22]. Triethylamine or K_2CO_3 is often used as a base to neutralize the HCl which is produced in the reaction. Both triethylamine and K_2CO_3 were tried in the attempt to synthesise methyl 6-(2-chloroacetoxy)hexanoate. K_2CO_3 was chosen because it made it easier to purify the substance and the yield tended to be higher. Flash chromatography on silica gave the product as a colorless oil and the yield was 91%. ^{1}H-NMR, ^{13}C-NMR, TLC and MS (EI) confirmed that the reaction had taken place and indicated a pure product.

Synthesis of 1,4,7-tris (tert-butylcarboxymethyl)- 10-[metyl-6(2-acetoxy) hexanoate] - 1,4,7,10-tetraazacyclododecan (DO3A-tert-butyl-MAH)(5)

Alkylation of DO3A-tert-butyl with alkyl halides is a widely used strategy for the preparation of DO3A derivates[23]. The reaction between DO3A-tert-butyl and methyl 6-(2-chloro-acetoxy)hexanoate was carried out in acetonitrile in the presence of K_2CO_3. The product was purified by flash chromatography and the yield was 23%. MS(ES^+) confirmed the reaction had taken place and four signals could be observed in the specter; m/z = 701, 645, 589 and 533. This is the molecule ion and the dissociation of one too three of the tert-butyl groups. ^{13}C-NMR showed the expected signals. The ^{1}H-NMR-specter of the substance was more difficult to read, but it confirmed the right number of hydrogens.

Synthesis of 1,4,7-tris (carboxymethyl)- 10-[metyl-6(2-acetoxy)hexanoate]- 1,4,7,10-tetraaza-cyclododecan (DO3A-MAH) (6)

The cleavage of tert-butyl esters is performed using TFA (see section 5.1.1). DO3A-tert-butyl-MAH was stirred in TFA and the solution was evaporated. The oil was solved in methanol and diethylether was added. The precipitate was filtered and washed with dietylether, but TLC and NMR indicated that the product was not pure. The substance was therefore submitted to flash chromatography on silica. MS(ES^+) confirmed the substance. The ^{1}H-NMR specter was not easy to interpret, but the integrals showed the right number of hydrogens. ^{13}C-NMR showed the expected signals, except the signals for the carbons in the cyclen. It appears like they came as a broad, multiple, low signal. The reason for this may be the multizwitterion properties of the

substance. Some of the nitrogens would be protonated and some of them not. This will probably affect the signals of the nearby carbons and give the result as appeared her.

Complexation of 1,4,7-tris (carboxymethyl)- 10-[metyl-6(2-acetoxy) hexanoate]- 1,4,7,10-tetraazacyclododecan with gadolinium (Gd-DO3A-MAH) (19)

Complexation of the ligand was performed using $GdCl_3 \times 6H_2O$. The reason $GdCl_3 \times 6H_2O$ was chosen and not Gd_2O_3 is that the complexation with Gd_2O_3 requires heating at 90-100°C over night. The stability of the complex under this condition was assumed to be low because the ester group which coordinated to gadolinium was exposed for hydrolysis. The disadvantage using $GdCl_3 \times 6H_2O$ is that you get an amount of inorganic salts in the product. The complexation was carried out according to the procedure in section 6.3 except this reaction was not heated. MS(ES^+) and TLC showed that only a small amount of the ligand was complexed.

5.1.3 Strategy 1 for synthesis of Gd-DO3A-ADA (Gadolinium-1,4,7-tris (carboxymethyl)- 10-[12-(2-acetamido)dodecanoic acid]-1,4,7,10-tetraazacyclododecan)

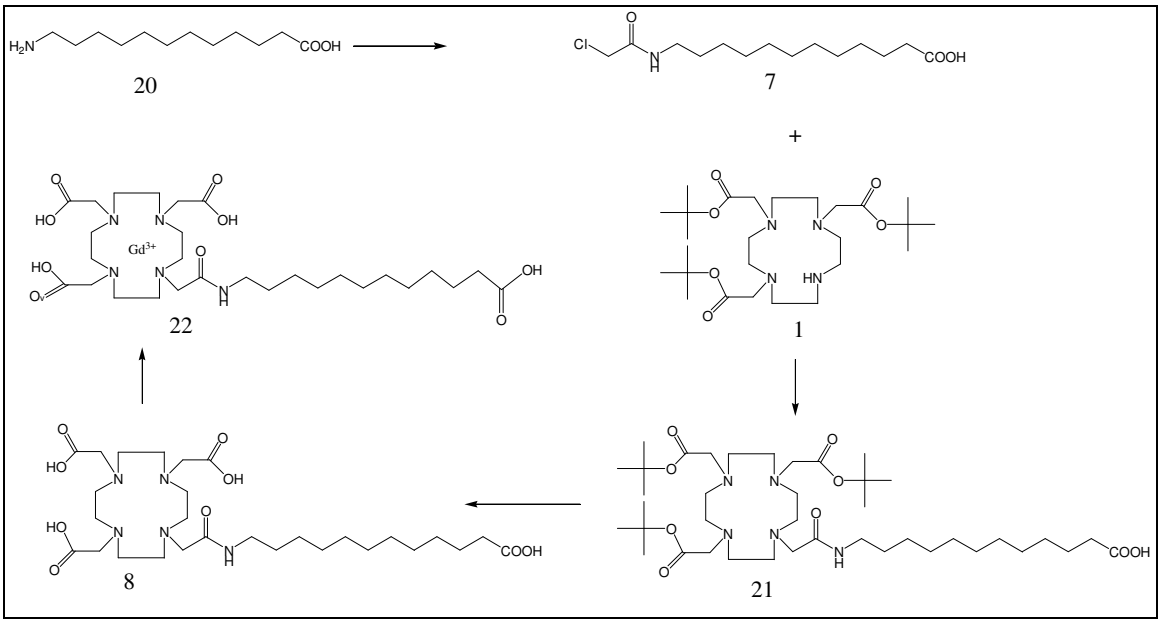


Figure 5.4: Strategy for synthesis of Gd-DO3A-ADA

It was attempt to use the same strategy to synthesize Gd-DO3A-ADA as was used for Gd-DO3A-MAH.

Synthesis of 12-(2-chloroacetamido)dodecanoic acid (7)

This reaction procedure was about the same as the method used for the synthesis of methyl 6-(2-chloroacetoxy)hexanoate. The problem was to find a solvent that would solve 12-aminododecanoic acid and not react with chloroacetyl chloride. Dioxane, dichloromethane, aetonitrile and THF were tried. THF gave the best result and was chosen. The product was purified by flash chromatography on silica and the yield was 60%. MS(EI) and NMR confirmed the substance.

Synthesis of 1,4,7-tris (tert-butylcarboxymethyl)- 10[12-(2-acetamido)dodecanoic acid] - 1,4,7,10-tetraazacyclododecan (DO3A-tert-butyl-ADA)(21)

The reaction was carried out under the same conditions as for the synthesis of DO3A-tert-butyl-MAH. TLC did not indicate that any reaction had occurred after 24 hours. The solvent was changed to chloroform, but no reaction occurred. The solvent was again changed to acetone and a small amount of NaI was added. There was still no reaction that occurred and therefore another strategy for the synthesis was chosen.

5.1.4 Strategy 2 for synthesis of Gd-DO3A-ADA (Gadolinium-1,4,7-tris (carboxymethyl)- 10-[12-(2-acetamido)dodecanoic acid]-1,4,7,10-tetraazacyclododecan)

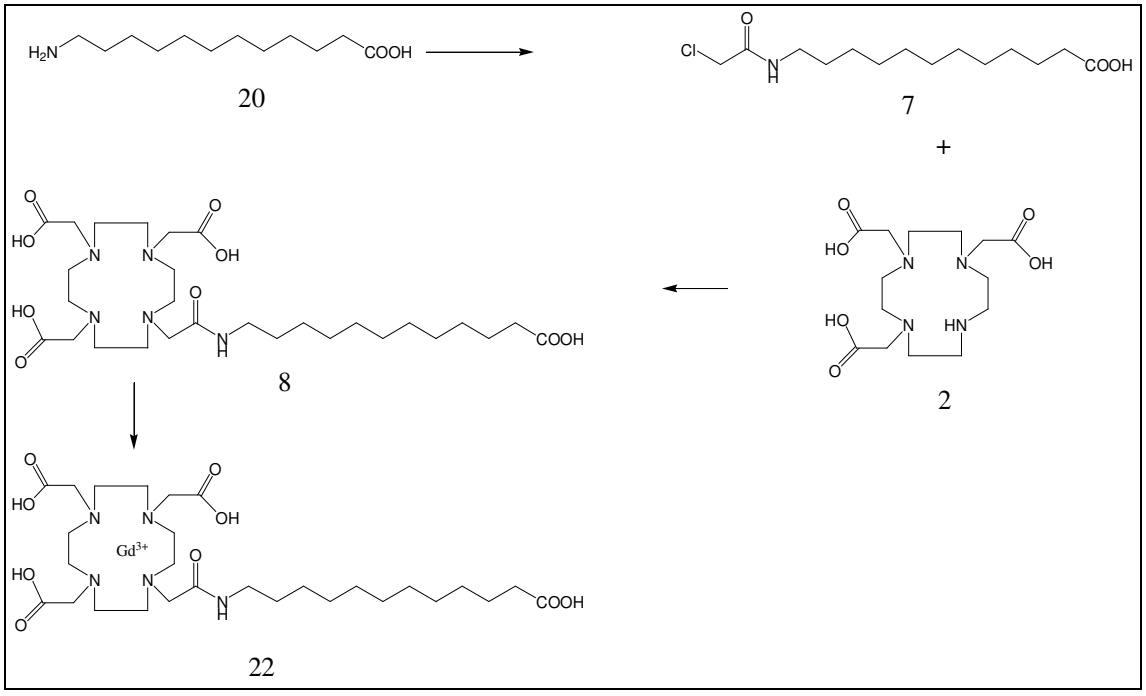


Figure 5.5: Strategy for synthesis of Gd-DO3A-ADA

Because alkylation of DO3A-tert-butyl did not succeed it was tried to alkylate DO3A directly. Similar reactions between DO3A and alkyl halides have been carried out earlier[24].

Synthesis of 1,4,7-tris (carboxymethyl)- 10[12-(2-acetamido)dodecanoic acid] - 1,4,7,10-tetraazacyclododecan (DO3A -ADA)(8)

Because it is a large difference in hydrofobicity between DO3A and 12-(2-acetamido) dodecanoic acid a solvent of methanol/ water had to be used. K_2CO_3 was added to neutralize HCl produced in the reaction and to provide that the secondary amine was not protonated. The product was evaporated in vacuum and submitted to flash chromatography on silica. The yield was 25%. NMR indicated the right product was synthesized. The signals for the carbons in the cyclen looked like the signals for the same carbons in DO3A-MAH (see synthesis of DO3A-MAH for discussion). The other signals showed up as expected. The ^{1}H-NMR specter was difficult to interpret. Anyway, integration of the signals gave the right number of protons. MS(ES^+) confirmed the substance.

Complexation of 1,4,7-tris (carboxymethyl)- 10[12-(2-acetamido)dodecanoic acid]- 1,4,7,10-tetraazacyclododecan with gadolinium (Gd-DO3A-ADA) (22)

DO3A-ADA was complexed with $GdCl_3 \times 6H_2O$ according to the procedure described in the experimental section. MS(ES^+) confirmed the complexation had taken place.

5.1.5 Strategy for synthesis of Gd-DO3A-MAD (Gadolinium 1,4,7-tris (carboxymethyl)- 10-[methyl 12-(2-acetamido) dodecanoate] - 1,4,7,10-tetraazacyclododecan)

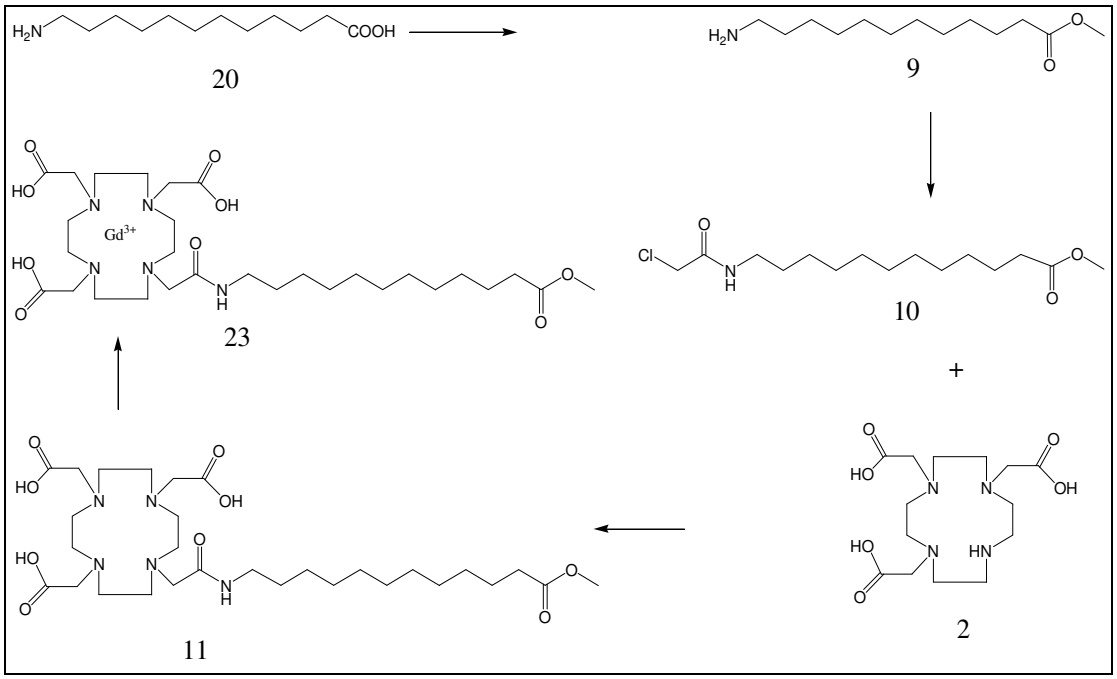


Figure 5.4: Strategy for synthesis of Gd-DO3A-MAD

As the synthesis of Gd-DO3A-ADA was successful the synthesis of Gd-DO3A-MAD was performed much the same way.

Synthesis of methyl-12-aminododecanoate (9)

The synthesis of methyl esters from amino acids and methanol is well known in the litterature[25]. Thionyl chloride was slowly added to a cooled solution of 12-aminododecanoic acid and methanol. The solution was evaporated in vacuum and purified by flash chromatography on silica. The yield was 92%. NMR and MS(ES^+) confirmed the substance.

Synthesis of methyl 12-(2-chloroacetamido)dodecanoate (10)

The synthesis was almost identical as for 12-(2-chloroacetamido)dodecanoic acid, except THF did not seem to be a good solvent. This was probably due to the increased hydrofobicity of methyl-12-aminododecanoate versus 12-aminododecanoic acid. Chloroform was chosen instead.

After filtration and evaporation the substance was purified by flash chromatography on silica. The yield was 62%. NMR and MS(ES^+) confirmed the substance.

Synthesis of 1,4,7-tris (carboxymethyl)- 10-[methyl 12-(2-acetamido)dodecanoate] - 1,4,7,10-tetraazacyclododecan (DO3A-MAD) (11)

This step in the synthesis was almost parallel as for DO3A-ADA, except the base was switched to $NaHCO_3$. The reason for this is that the high temperature and K_2CO_3 seemed to hydrolyse the methyl ester. The yield of the reaction (16%) was something lower than for DO3A-ADA. The reason for this may be the weaker base which would not deprotonate the secondary amine of DO3A in the same way as K_2CO_3. ^{13}C-NMR for the substance was quite similar the specter for DO3A-ADA and contained in addition a clear signal for the carbon in the methyl ester group. ^{1}H-NMR was also almost identical to the specter for DO3A-ADA, but also here was the singlet from the hydrogens in the methyl ester group very clear. MS(ES^+) confirmed the substance.

Complexation of 1,4,7-tris (carboxymethyl)- 10[methyl 12-(2-acetamido)dodecanoic acid]-1,4,7,10-tetraazacyclododecan with gadolinium (Gd-DO3A-MAD) (23)

DO3A-ADA was complexed with $GdCl_3 \times 6H_2O$ according to the procedure described in the experimental section. MS(ES^+) confirmed the complexation had taken place.

5.1.6 Strategy for synthesis of Gd-DO3A-APP

(Gadolinium 1,4,7-tris (carboxymethyl)- 10-[(L)-2-(2-acetamido)-3-phenyl-propanoic acid] - 1,4,7,10-tetraazacyclododecan)

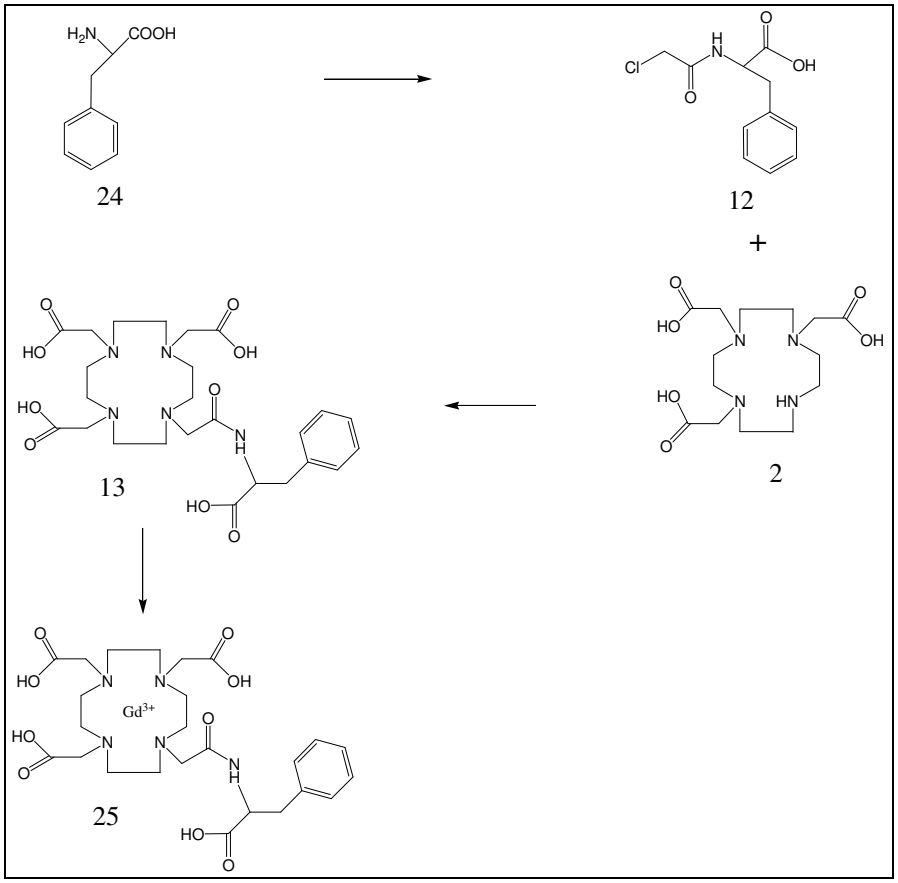


Figure 5.5; Strategy for synthesis of Gd-DO3A-APP

The strategy for the synthesis of Gd-DO3A-APP was similar to the strategy used for Gd-DO3A-MAD and Gd-DO3A-ADA.

Synthesis of (L)-2-(2-chloroacetamido)-3-phenylpropanoic acid (12)

The synthesis of (L)-2-(2-chloroacetamido)-3-phenylpropanoic acid has earlier been described in the literature[26]. Chloroacetyl chloride was added to a solution of L-phenylalanine and acetonitrile under nitrogen. The solution was put on reflux until it became clear. After evaporation of the acetonitrile, TLC did not indicate any impurities. NMR and MS(ES^+) also indicated the right

substance was synthesized and indicated a pure product. No more work up of the substance was carried out. The yield was 100%.

Synthesis of 1,4,7-tris (carboxymethyl)- 10-[(L)-2-(2-acetamido)-3-phenylpropanoic acid] - 1,4,7,10-tetraazacyclododecan (DO3A-APP)(13)

The reaction between DO3A and (L)-2-(2-acetamido)-3-phenylpropanoic acid was carried out in a solution of water, methanol and K_2CO_3. The solvent was evaporated off and the product was submitted to flash chromatography on silica, giving a yield of 56%. ^{13}C-NMR showed the expected signals. The carbons in the cyclen appeared as described for DO3A-MAH. As for the other DO3A-derivates, either ^{1}H-NMR for DO3A-APP was not easy to interpret. Anyway, integration of the specter showed the right number of hydrogens. MS(ES^+) confirmed the substance.

Complexation of 1,4,7-tris (carboxymethyl)- 10[(L)-2-(2-acetamido)-3-phenylpropanoic acid]-1,4,7,10-tetraazacyclododecan with gadolinium (Gd-DO3A-APP) (25)

DO3A-APP was complexed with $GdCl_3 \times 6H_2O$ according to the procedure described in the experimental section. MS(ES^+) confirmed the complexation had taken place.

5.1.7 Strategy for synthesis of Gd-DO3A-MAP

(Gadolinium 1,4,7-tris (carboxymethyl)- 10-[(L)-methyl 2-(2-acetamido)-3-phenylpropanoate] - 1,4,7,10-tetraazacyclododecan)

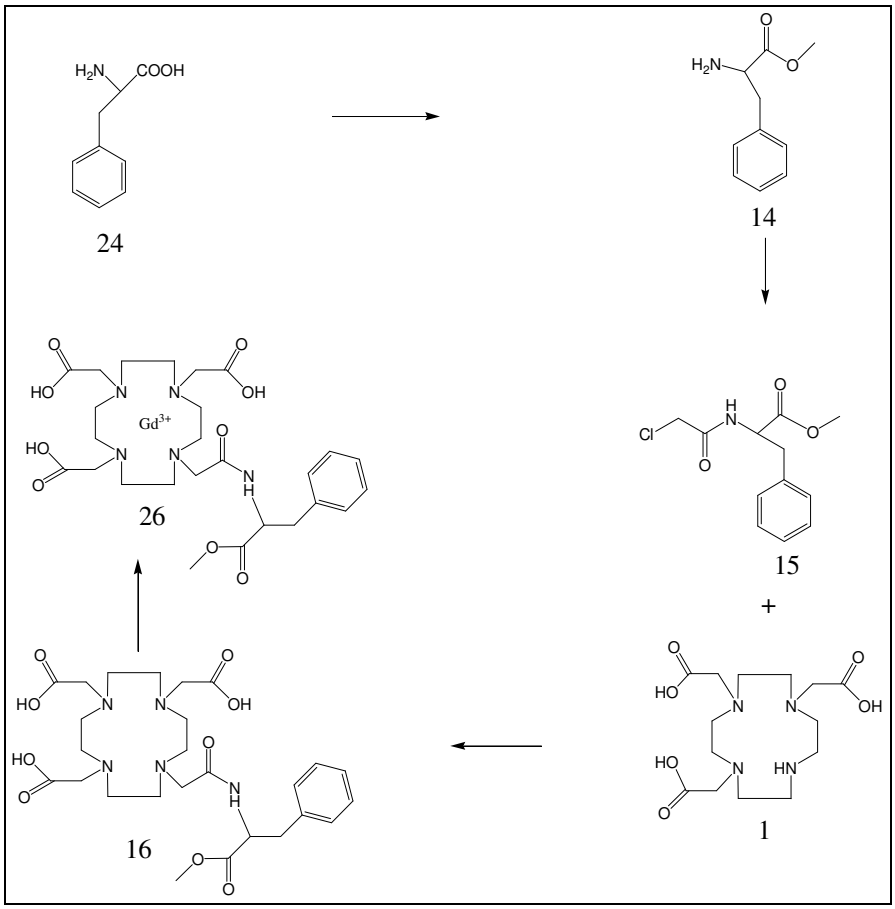


Figure 5.5; Strategy for synthesis of Gd-DO3A-MAP

The strategy for the synthesis of Gd-DO3A-MAP was similar to the strategy used for Gd-DO3A-MAD, Gd-DO3A-APP and Gd-DO3A-ADA.

Synthesis of (L)-methyl-2-amino-3-phenylpropanoate(14)

As earlier mentioned the synthesis of a methyl ester from an amino acid and methanol is well known in the litterature[25]. Thionyl chloride was slowly added to a cooled solution of L-phenylalanine and methanol. The solution was evaporated in vacuum and purified by flash chromatography on silica and the yield was 93%. NMR and MS(ES^+) confirmed the substance.

Synthesis of (L)-methyl 2-(2-chloroacetamido)-3-phenylpropanoate(15)

Chloroacetyl chloride was added to a cooled solution of K_2CO_3 and (L)-methyl-2-amino-3-phenylpropanoate in 25ml dichloromethane and 10ml acetonitrile. After stirring over night, the solution was filtered, evaporated in vacuum and purified by flash chromatography on silica. NMR and MS(ES^+) confirmed the substance. The yield was 56%.

Synthesis of 1,4,7-tris (carboxymethyl)- 10-[(L)-methyl 2-(2-acetamido)-3-phenyl-propanoate]-1,4,7,10-tetraazacyclododecan (DO3A-MAP)(16)

The alkylation of DO3A was performed similar to the reaction used for the synthesis of DO3A-MAD. TLC indicated that it was two substances in the end product, but it was not easy to see because of the relatively like retention time of the substances (0.47 versus 0.45). HPLC clearly showed a product consisting of two substances. The one had the same eluation time as DO3A-APP. It did not succeed to purify the product, neither flashing on silica nor using an ion exchange column.

Complexation of 1,4,7-tris (carboxymethyl)- 10-[(L)-methyl 2-(2-acetamido)-3-phenyl-propanoate]- 1,4,7,10-tetraazacyclododecan with gadolinium (Gd-DO3A-MAP)(26)

Even the DO3A-MAP was not pure it was complexed with $GdCl_3 \times 6H_2O$ according to the procedure described in the experimental section. MS(ES^+) confirmed the complexation had taken place, but it also indicated that Gd-DO3A-APP was formed.

5.1.8 Complexation of DTPA with gadolinium

Gd-DTPA was prepared from $GdCl_3 \times 6H_2O$ and diethylenetriaminepentaacetic acid as described in the experimental section. MS(ES^+) confirmed the substance.

5.1.9 Conclusion

Three complexes have been prepared (Gd-DO3A-ADA, Gd-DO3A-MAD and Gd-DO3A-APP). One ligand (Gd-DO3A-MAH) has been synthesised and characterized but the complexation failed. Due to lack of time no more of the ligand was synthesized. The last ligand, Gd-DO3A-MAP, was synthesised but it was not pure. The purification failed.

5.2 Binding to bovine serum albumin

Albumin is a well known carrier for drugs and other small molecules. Its structure has been known since 1975[27]. Albumin consist of a single peptide chain of 585 amino acids and has a molecular weight of approximately 66 300 Da[27, 28]. It is mostly α-helical and consist of three structurally homologues domains and has a shape like a heart[29]. (Figure 5.6)

Figure 5.6: Schematic structure of albumin

The three domains can be further divided into subdomains A and B. Those subdomains are often used to explain were different binding sites on albumin are located. Sudlow et al have described two important sites which binds several drugs[30, 31]. Those principal regions of ligand binding to human serum albumin are located in hydrophobic cavities in subdomains IIA and IIIA[32]. Bhattacharya et al have described several binding sites for fatty acids[18].

5.2.1 Affinity for albumin

The typical dose of a gadolinium based contrast agent injected is 0.1mmol/kg body weight. For two contrast agents, Magnevist and Gadovist, the plasma concentration will reach a maximum of respectively 2.0mM and 0.59mM[19]. The plasma concentration of albumin is 0.67mM. In the determination of the albumin binding it was desirable to keep the concentration of the complex lower than that of albumin. At the same time it was desirable to use a concentration that is close to plasma concentration of the contrast agents used today. A 0.50mM solution of the complexes was chosen. The albumin binding of Gd-DTPA was also tried to be measured, but it failed to develop a HPLC method for this substance. From the literature this value was found to be close to zero[33].

The binding of the complexes Gd-DO3A-ADA, Gd-DO3A-MAD and Gd-DO3A-APP to albumin was measured.

Table 5.1; Albumin binding of the Gd(III) complexes.

Substance	Parallel	Albumin binding	Average
Gd-DO3A-ADA	1	18.6 %	**19.2± 0.9 %**
	2	18.4 %	
	3	20.5 %	
Gd-DO3A-APP	1	-1.5 %	**0 %**
	2	0 %	
	3	-0.6 %	
Gd-DO3A-MAD	1	58.5 %	**60.0±1.4 %**
	2	61.8 %	
	3	59.6 %	

5.2.2 Conclusion

The affinity upon albumin for the three complexes was measured. The methyl ester, Gd-DO3A-MAD, had the highest affinity for albumin. Gd-DO3A-ADA also had a low degree of albumin binding, while Gd-DO3A-APP did not show any degree of binding at all.

5.3 Relaxivity

Determinations of the T_1–relaxivities of the Gd(III) complexes were carried out in phosphate buffer solutions and in BSA, phosphate buffer solutions. Gd-DTPA was used as a control.

5.3.1 T_1–relaxivities of the Gd(III) complexes.

The T_1–relaxivities (r_1) (20MHz, 40°C, pH 7.4) of Gd-DO3A-ADA, Gd-DO3A-MAD and Gd-DO3A-APP were measured. The results are summarized in table 5.2.

Table 5.2; Relaxivity of the Gd(III) complexes.

Substance	T1 (ms)			R1 ($mM^{-1}s^{-1}$)
Phosphate buffer pH 7.4	3000	3000	3000	
Gd-DTPA 10mM in buffer	26.9	27.8	27.1	**3.63**
Gd-DTPA 25mM in buffer	9.5	10.2	10.5	**3.96**
Gd-DTPA 25mM in albumin	8.0	8.4	8.0	**4.90**
Gd-DO3A-ADA 10mM in buffer	22.7	25.3	24.8	**4.09**
Gd-DO3A-ADA 10mM in albumin	16.9	16.8	17.1	**5.87**
Gd-DO3A-ADA 20mM in albumin	8.5	8.8	8.8	**5.73**
Gd-DO3A-APP 10mM in buffer	22.2	25.5	26.5	**4.01**
Gd-DO3A-APP 10mM in albumin	23.0	24.2	23.4	**4.22**
Gd-DO3A-APP 20mM in albumin	12.1	12.2	12.8	**4.03**
Gd-DO3A-MAD 10mM in buffer	23.6	25.2	25.9	**3.98**
Gd-DO3A-MAD 2.5mM in albumin	46.7	46.9	48.2	**8.33**
Gd-DO3A-MAD 5mM in albumin	24.9	24.0	24.8	**8.07**
Gd-DO3A-MAD 10mM in albumin	13.2	13.1	12.9	**7.62**
Gd-DO3A-MAD 1.25mM in albumin 10%	48.2	47.8	47.7	**16.47**

5.3.2 Conclusion

The T_1-relaxivity of the complexes has been measured. Gd-DTPA showed values which can be related to results in the literature[8, 34, 35]. Gd-DO3A-ADA, Gd-DO3A-MAD and Gd-DO3A-APP had relaxivities as expected. In the buffer solution all complexes had relaxivities close to 4.0 $mM^{-1}s^{-1}$. From the literature, complexes of DO3A-derivates tend to have relaxivities close to this value[8]. As expected an increase in relaxivity was observed in the albumin solutions where the

complexes had any degree of albumin binding. From table 5.2 an increase in the relaxivity of Gd-DO3A-ADD and Gd-DO3A-MAD in albumin could be seen when the concentration of them decreased. The assumed explanation for this is the excess of compounds in proportion to albumin in the solution. The ratio of bound and unbound gadolinium complex to albumin is increased when the concentration of the complexes decreases.

5.4 Displacement

As discussed in section 5.2, there are several binding sites on albumin. To confirm the protein binding of Gd-DO3A-MAD was the reason for the increased relaxivity, different substances that were expected to displace the complex were added to the HSA-complex solution and the relaxivity was measured. As described, two sites on subdomain IIA and IIIA binds a lot of drugs. Two drugs with high affinity for those sites are warfarin(IIA) and oxazepam(IIIA)[27,36]. Those were picked out. As the assumed protein binding part of the molecule, Gd-DO3A-MAD, is a fatty acid methyl ester, a fatty acid was also attempt as a displacer. If one of the two substances with a known binding site on albumin would involve a decreased relaxivity, this could also tell where the complex was bound.

5.4.1 Relaxivity of Gd-DO3A-MAD with added displacers

Two strategies were carried out in the attempt to displace Gd-DO3A-MAD. The first attempt was to add the displacers to a 2.5mM solution of Gd-DO3A-MAD in 4% BSA and measure the relaxivity. The second attempt was to first add the displacers to an 8% BSA solution and then add an equal volume of 5mM Gd-DO3A-MAD (making a final solution of 2.5mM Gd-DO3A-MAD in 4% BSA) and measure the relaxivity. The substances added were warfarin, oxazepam and caprylic acid. The results are summarized in table 5.3 and 5.4

Strategy 1

Table 5.3; Relaxivity of Gd-DO3A-MAD in albumin and with added substances

Substance	T1 (ms)			R1 (mM-1s-1)
Phosphate buffer pH 7.4	3000	3000	3000	
Gd-DO3A-MAD 2.5mM in albumin	46.9	48.6	48.3	**8.21**
Gd-DO3A-MAD 2.5mM in albumin + oxazepam 5mM	47.8	47.9	46.7	**8.29**
Gd-DO3A-MAD 2.5mM in albumin + warfarin 5mM	48.9	48.6	47.9	**8.12**
Gd-DO3A-MAD 2.5mM in albumin + caprylic acid 5mM	48.5	48.6	48.0	**8.14**

Strategy 2

Table 5.4; Relaxivity of Gd-DO3A-MAD in albumin and with added substances

Substance	T1 (ms)			R1 (mM-1s-1)
Phosphate buffer pH 7.4	3000	3000	3000	
Gd-DO3A-MAD 2.5mM in albumin	46.7	46.9	48.2	**8.33**
Gd-DO3A-MAD 2.5mM in albumin + oxazepam 5mM	46.4	47.6	45.8	**8.45**
Gd-DO3A-MAD 2.5mM in albumin + warfarin 5mM	45.6	47.0	46.8	**8.48**
Gd-DO3A-MAD 2.5mM in albumin + caprylic acid 5mM	79.5	80.3	80.5	**4.86**

5.4.2 Conclusion

In the first strategy it did not succeed to observe any decrease in the relaxation time. By using the second strategy a clear decrease in the relaxivity was seen when caprylic acid was added. This could indicate that Gd-DO3A-MAD binds to the same binding site as the caprylic acid. Another explanation may be that binding of caprylic acid to albumin could alter the structure of albumin, and of that reason prevent the binding of Gd-DO3A-MAD.

6 EXPERIMENTAL

6.1 Chemicals and equipment

6.1.1 Chemicals

Chemicals were obtained from Aldrich Chemical Co.Inc.,USA, Fluka Chemie AG, Switzerland and Merck KGaA, Germany and used as received.

6.1.2 Equipment

NMR-spectra were obtained on Bruker Spectrospin Avance DPX 300 (Bruker GmbH, Germany). All spectra were recorded at 25°C.

ES mass spectra were recorded on a Q-tof 2 (Micromass Ltd, UK). EI mass spectra were obtained at Fisions VG ProSpec Q, 70eV (Micromass Ltd, UK)

Relaxation measurements were performed at 40°C and 20MHz on a Bruker Minispec mq 20 NMR Analyzer (Bruker Analytik GmbH, Germany)

HPLC analyses were performed on a Hewlett Packard 1100 instrument (Hewlett Packard GmbH, Germany). The column was a Phenomenax C-18 reversed phase (250mm×2mm, 5µm) and the detector was a diode UV detector (205 nm was used).

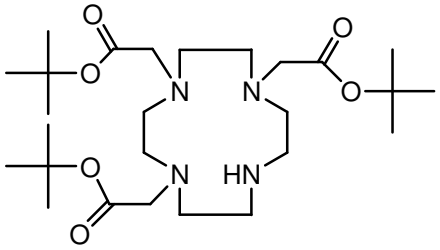
6.1.3 Chromatography

For analysis with TLC, TLC sheets with silica gel 60 F_{254} were used (DC-Alufolien 20×20 cm Kiselgel 60 F_{254}, Merck). To recover the substances elementary iod or a solution of $KMnO_4$ in water (1% w/w) were used.

Silica gel 60 with particle size 0.04-0.063mm was used for flash chromatography. (Silica gel 60, Fluka)

6.2 Synthesis procedures

1,4,7-tris(carboxymethyl-tert-butylester)-1,4,7,10-tetraazacyclodecane (DO3A-tert-butyl)



1

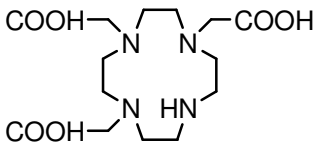
Cyclen (35g, 0.20mol) and sodiumacetate (50g, 0.61mol) were dissolved in 600 ml dimethyl-acetamide (DMA). Bromoacetate (118.9g, 0.61mol) was solved in another 150 ml of DMA and added to the first solution. The mixture was stirred for 19 days at room temperature and the precipitate was then collected by filtration. The filtrate was concentrated to afford a second crop of the product. The combined crops were dissolved in chloroform and washed with water. The chloroform was evaporated off and the residue was a yellow oil. The addition of ethyl acetate to the oil gave a white solid which was collected by filtration and washed with ether. The yield was 48.3g (40%).

^{13}C NMR (300MHz, $CDCl_3$): δ 27.98, 28.01, 47.30, 48.93, 51.08, 57.93, 81.41, 169.39, 170.29

MS (ES^+) (Mw=514): 515 ($M+H^+$), 459($M-C_4H_9$), 403($M-2C_4H_9$), 347($M-3C_4H_9$)

(NMR-specter: Appendix 1.1)

1,4,7-tris(carboxymethyl)-1,4,7,10-tetraazacyclodecane (DO3A)



2

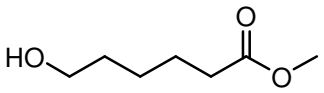
DO3A-tert-butyl (5.01g, 9.8mmol) was added 45 ml of triflouroacetic acid (600mmol). The solution was stirred at room temperature for 16 hours. The solvent was evaporated and the residue was solved in 10 ml of methanol. 100 ml diethyleter was slowly added under vigorous stirring. The precipitate was collected by filtration and washed with diethylether. The product was dried under vacuum. The yield was 96% (3.26g)

^{13}C NMR (300MHz, D_2O): δ 42.96, 48.37, 49.64, 52.37, 53.82, 56.28, 170.45, 175.35

MS (ES^+) (Mw=346): 347 ($M+H^+$)

(NMR-specter: Appendix 1.2)

Methyl-6-hydroxyhexanoate



3

2 ml 12 M H_2SO_4 was added dropwise to a stirred solution of ε-caprolactone (11.4g, 0.10mol) in 250 ml methanol. The mixture was refluxed for 22 hours and then cooled to room temperature. The solvent was evaporated in vacuum. The residue was dissolved in 100ml water and neutralized with solid $NaHCO_3$. The water was extracted with 4×25 ml diethyleter. Extracts was dried with $MgSO_4$, filtered and evaporated. The residue was a colorless oil which was vacuum distilled (Boiling point 102-106°C and pressure 25mBar). The yield was 4.1g (28%).

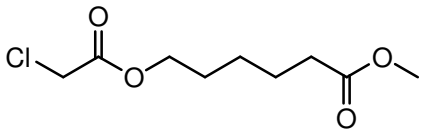
^{1}H NMR (300MHz, $CDCl_3$): δ 1.22-1.34 (m, 2H), 1.42-1.61 (m, 4H), 2.22 (t, 7.4 Hz, 2H), 2.38 (s, 1H), 3.51 (t, 6.5 Hz, 2H), 3.56 (s, 3H)

^{13}C NMR (300MHz, $CDCl_3$): δ 24.55, 25.21, 32.19, 33.90, 51.42, 62.41, 174.16

MS (EI) (Mw=146): 146 (M), 128 (M-H_2O)

(NMR-specter: Appendix 2)

Methyl 6-(2-chloroacetoxy)hexanoate



4

A cooled solution of 10 ml dichloromethane was added chloroacetyl chloride (0.39g, 3.4mmol) and K_2CO_3 (0.62g, 4.5mmol). Methyl-6-hydroxyhexanoate (0.50g, 3.4mmol) was slowly dripped to the solution. The ice bath was removed and the solution stirred at room temperature for 2 hours. The solution was then filtered and evaporated in vacuum. The residue was submitted to flash chromatography on silica (CH_2Cl_2:$CHCl_3$, 1:1). The yield was 0.69g (91%) of a colorless oil.

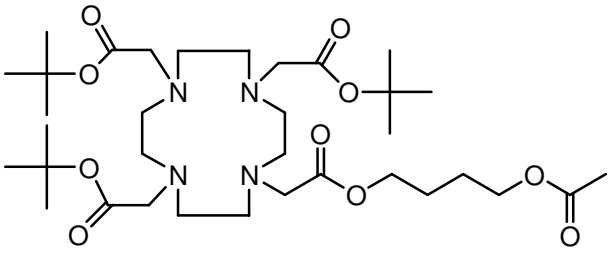
^{1}H NMR (300MHz, $CDCl_3$): δ 1.30-1.48 (m, 2H), 1.59-1.77 (m, 2H), 2.31 (t, 7.4 Hz, 2H), 3.66 (s, 3H), 4.04 (s, 2H), 4.18 (t, 6.6 Hz, 2H)

^{13}C NMR (300MHz, $CDCl_3$): δ 24.41, 25.30, 28.09, 33.77, 40.84, 51.48, 65.95, 167.29, 173.81

MS (EI) (Mw=222): 222 (M), 187 (M-Cl)

(NMR-specter: Appendix 3)

1,4,7-Tris (tert-butylcarboxymethyl)- 10-[metyl-6(2-acetoxy)hexanoate]- 1,4,7,10-tetraazacyclododecan



5

Metyl-6(2-chloroacetoxy)hexanoate (0.45g, 2mmol), DO3A-tert-butyl (0.89g, 1.5mmol) and K_2CO_3 (0.48g, 3.5mmol) was added to 15 ml acetonitrile. The solution was stirred at reflux for 16 hours. The solution was filtered and the solvent was evaporated. The residue was submitted to flash chromatography on silica (CH_2Cl_2: MeOH: NH_3 (25%), 8:2:0.1). The yield was 0.35g (23%) of a yellow oil.

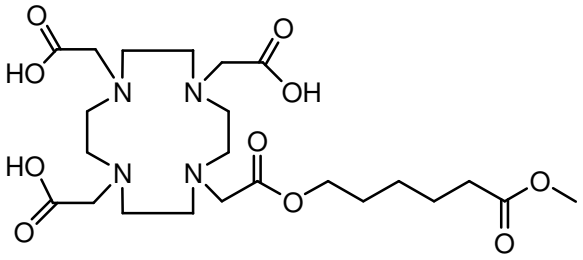
^{1}H NMR (300MHz,$CDCl_3$): δ 1.23-1.36 (m, 3H), 1.40 (s, 27H), 1.53-1.68 (m, 5H), 1.92-2.20 (m, 3H), 2.22-2.31 (m, 4H), 2.32-2.61 (m, 5H), 2.63-3.49 (m, 12H), 3.42 (s, 2H), 3.62 (s, 3H), 4.04 (t, 6.7 Hz, 2H)

^{13}C NMR (300MHz, $CDCl_3$): δ 24.43, 25.43, 27.87, 27.95, 28.15, 28.21, 33.73, 51.49, 54.85, 55.62, 55.68, 64.92, 81.86, 172.91, 172.99, 173.64, 173.76

MS (ES^+) (Mw=700): 701($M+H^+$), 645($M-C_4H_9$), 589($M-C_4H_9$), 533($M-C_4H_9$)

(NMR-specter: Appendix 4)

1,4,7-Tris (carboxymethyl)- 10-[metyl-6(2-acetoxy)hexanoate]- 1,4,7,10-tetraaza-cyclododecan



6

1,4,7-Tris (tert-butylcarboxymethyl)- 10-[metyl-6(2-acetoxy)hexanoate]- 1,4,7,10-tetraaza-cyclododecan (0.35g, 0.5mmol) was dissolved in 5 ml TFA. The solution was stirred at room temperature for 3 hours. The solvent was evaporated in vacuum and the residue (a yellow oil) was solved in 5 ml methanol. 50 ml diethylether was slowly added under severe stirring. After a while a white precipitate occurred. The solution was filtered on a schinter funnel and washed with diethylether. The white solid was then dried in vacuum and purified by flash chromatography on silica (MeOH: H_2O: NH_3 (25%), 9:1:0.1). The yield was 0.17g (66%).

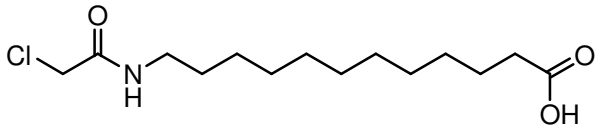
^{1}H NMR (300MHz, D_2O): δ 1.30-1.42 (m, 2H), 1.51- 1.73 (m, 4H), 1.78-3.33 (m, 23H), 3.33-3.63 (m, 3H), 3.66 (s, 3H), 4.08-4.48 (m, 2H)

^{13}C NMR (300MHz, D_2O): δ 24.32, 25.08, 27.73, 33.95, 48.45, 52.54, 53.13, 56.60, 59.14, 59.40, 67.91, 177.90, 178.19, 180.23, 180.37

MS (ES^+) (Mw=532): 533 ($M+H^+$)

(NMR-specter: Appendix 5)

12-(2-chloroacetamido)dodecanoic acid



7

A solution of 12-aminododecanoic acid (0.43g, 2mmol) and K_2CO_3 (0.56g, 4mmol) in 15 ml terahydrofurane (THF) was cooled on an ice bath. Chloroacetyl chloride (165ml, 2mmol) was slowly added. The ice bath was removed and the solution was stirred at room temperature for 3 hours. The solution was filtered and evaporated. The white solid that appeared was submitted to flash chromatography on silica (CH_2Cl_2: MeOH, 9:1) and the yield was 0.35g (60%) of a white solid.

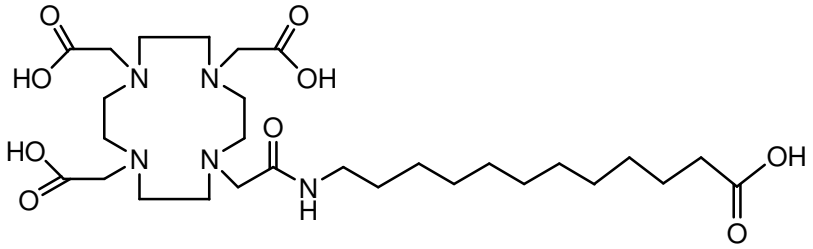
^{1}H NMR (300MHz, $CDCl_3$): δ 1.21-1.38 (m, 14H), 1.44-1.72 (m, 4H), 2.33 (t, 7.5 Hz, 2H), 3.30 (q, 6.9 Hz, 2H), 4.07 (s, 2H), 6.62 (bs, 1H)

^{13}C NMR (300MHz, $CDCl_3$): δ 24.65, 26.73, 28.96, 29.13, 29.24, 29.28, 29.35, 33.98, 39.93, 42.66, 166.02, 179.36

MS (EI) (Mw=291): 291 (M), 273 (M-H_2O), 256 (M-Cl)

(NMR-specter: Appendix 6)

1,4,7-Tris (carboxymethyl)- 10-[12-(2-acetamido)dodecanoic acid]- 1,4,7,10-tetraaza-cyclododecan



8

12-(2-chloroacetamido)dodecanoic acid (1.00g, 3.43mmol), DO3A (1.00g, 2.89mmol) and K_2CO_3 (2.50g, 18.00mmol) was added to a solution of 30 ml water and 60 ml methanol. The solution was stirred at 60°C for 16 hours and then evaporated. The residue was purified by flash chromatography on silica (MeOH: H_2O:NH_3 (25%), 9:1:0.1). The yield was 0.44g (25%) of a white solid.

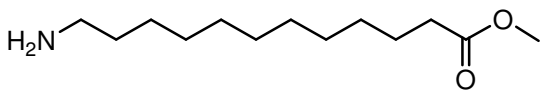
^{1}H NMR (300MHz, MeOD): δ 1.29 (s, 14H), 1.42-1.67 (m, 4H), 1.93-3.94 (m, 28H)

^{13}C NMR (300MHz, MeOD): δ 28.87, 28.15, 30.22, 30.41, 30.53, 30.61, 30.65, 36.96, 40.92, 54.09, 58.21, 60.35, 60.46, 174.55, 179.58, 180.16, 180.25

MS (ES^+) (Mw=601): 602 ($M+H^+$), 640 ($M+K^+$)

(NMR-specter: Appendix 7)

Methyl-12-aminododecanoate



9

12-aminododecanoic acid (0.86g, 4mmol) was solved in 25 ml methanol and cooled on an ice bath. $SOCl_2$ (0.33ml, 4.3mmol) was slowly added under stirring. The ice bath was removed and the solution stirred overnight. The solvent was evaporated and the residue was submitted to flash chromatography on silica (CH_2Cl_2: MeOH: $NH_3$25%, 7:3:0.1). The yield was 0.84g (92%) of a white solid.

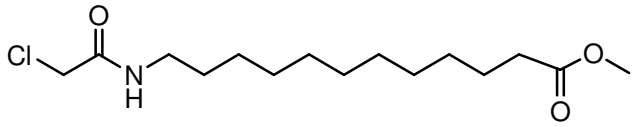
^{1}H NMR (300MHz, MeOD): δ 1.28-1.48 (m, 14H), 1.56-1.76 (m, 4H), 2.34 (t, 7.4 Hz, 2H), 2.95 (t, 7.6 Hz, 2H), 3.68 (s, 3H)

^{13}C NMR (300MHz, MeOD): δ 26.05, 27.49, 28.61, 30.21, 30.24, 30.38, 30.49, 30.57, 34.83, 40.83, 52.00, 176.01

MS (ES^+) (Mw=229): 230 ($M+H^+$)

(NMR-specter: Appendix 8)

Methyl 12-(2-chloroacetamido)dodecanoate



10

Methyl 12-dodecanoate (0.46g, 2mmol) was dissolved in 15 ml chloroform and K_2CO_3 (0.28g, 2mmol) was added. The solution was cooled on an ice bath and chloroacetyl chloride (165ml, 2mmol) was slowly added. The ice bath was removed and the solution was stirred at room temperature for 2 hours. The solution was filtered and evaporated. The white solid that appeared was purified by flash chromatography on silica (Hexane: Ethylacetate, 6:4) and the yield was 0.38g (62%) of a white solid.

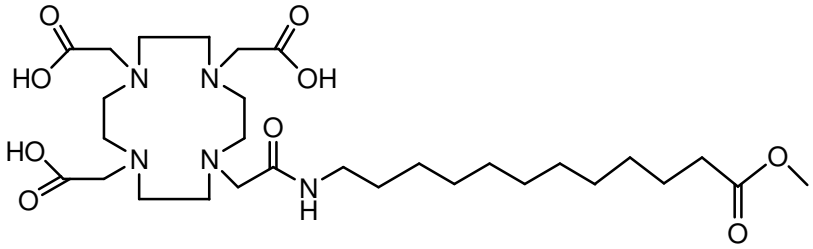
^{1}H NMR (300MHz, $CDCl_3$): δ 1.23-1.35 (m, 14H), 1.47-1.70 (m, 4H), 2.30 (t, 7.5 Hz, 2H), 3.30 (q, 7.1 Hz, 2H), 3.66 (s, 3H), 4.08 (s, 2H)

^{13}C NMR (300MHz, $CDCl_3$): δ 24.91, 26.74, 29.09, 29.17, 29.33, 29.39, 34.09, 40.06, 42.55, 51.47, 166.44, 174.48

MS (ES^+) (Mw=305): 306 ($M+H^+$)

(NMR-specter: Appendix 9)

1,4,7-Tris (carboxymethyl)- 10-[methyl 12-(2-acetamido)dodecanoate] - 1,4,7,10-tetraazacyclododecan



11

Methyl 12-(2-chloroacetamido)dodecanoate (0.46g, 1.5mmol), DO3A (0.52g, 1.5mmol) and $NaHCO_3$ (0.63g, 7.5mmol) was dissolved in 10 ml water and 20 ml methanol. The solution was stirred at 70°C for 16 hours and the solvent was evaporated. The residue was submitted to flash chromatography on silica (MeOH: H_2O: NH_3 (25%), 9:1:0.1). The yield was 0.15g (16%) of a white solid.

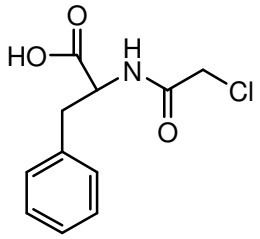
^{1}H NMR (300MHz, D_2O): δ 1.28 (s, 14H), 1.41-1.67 (m, 4H), 1.98-3.64 (m, 28H), 3.66 (s, 3H)

^{13}C NMR (300MHz, D_2O): δ 26.45, 28.24, 30.22, 30.47, 30.64, 30.84, 30.89, 35.74, 41.59, 49.97, 53.96, 54.29, 58.98, 60.71, 60.86, 175.56, 178.88, 181.98

MS (ES^+) (Mw=615): 616 ($M+H^+$), 638 ($M+Na^+$)

(NMR-specter: Appendix 10)

(L)-2-(2-chloroacetamido)-3-phenylpropanoic acid



12

L-phenylalanine (0.50g, 3.0mmol) was added to 10 ml acetonitrile under nitrogen. Chloroacetyl chloride (253ml, 3.1mmol) was added and the solution was put on reflux until the solution became clear. The solvent was evaporated and a yellow oil appeared. The oil crystallized after a while and the yield was 0.72g (100%)

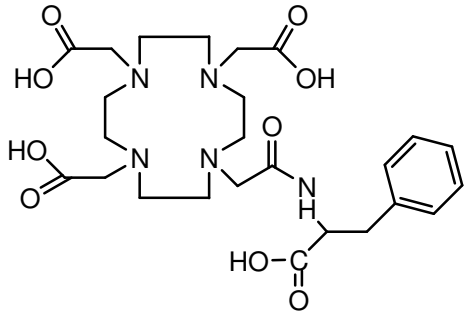
^{1}H NMR (300MHz, MeOD): δ 2.89-3.00 (q, 8.4 Hz, 1H), 3.10-3.20 (q, 5.1Hz, 1H), 3.93 (s, 2H), 4.57-4.64 (q, 5.1Hz, 1H), 7.09-7.24 (m, 5H)

^{13}C NMR (300MHz, MeOD): δ 38.19, 42.95, 55.29, 127.93, 129.50, 130.35, 138.06, 168.92, 174.03

MS (ES^+) (Mw=241): 242 ($M+H^+$)

(NMR-specter: Appendix 11)

1,4,7-Tris (carboxymethyl)- 10-[(L)-2-(2-acetamido)-3-phenylpropanoic acid]- 1,4,7,10-tetraazacyclododecan



13

2-(2-chloroacetamido)-3-phenylpropanoic acid (0.96g, 4mmol), DO3A (1.38g, 4mmol) and K_2CO_3 (3.34 g, 24mmol) was dissolved in 10 ml water and 20 ml methanol. The solution was stirred at reflux for 16 hours and the solvent was evaporated. The residue was submitted to flash chromatography on silica (MeOH: H_2O: NH_3 (25%), 9:1:0.1). The yield was 1.23g (56%) of a white solid.

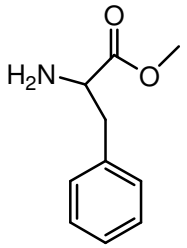
^{1}H NMR (300MHz, D_2O): δ 1.36-3.01 (m, 22H), 3.18 (s, 2H), 3.24-3.60 (m, 2H), 4.37-4.53 (m, 1H), 7.05-7.25 (m, 5H)

^{13}C NMR (300MHz, D_2O): δ 37.54, 47.23, 51.92, 55.79, 56.12, 57.98, 58.43, 126.18, 128.22, 128.65, 137.75, 172.45, 177.11, 178.78, 179.39

MS (ES^+) (Mw=551): 552 ($M+H^+$), 574 ($M+Na^+$), 590 ($M+K^+$)

(NMR-specter: Appendix 12)

(L)-Methyl-2 amino-3-phenylpropanoate



14

L-phenylalanine (2.07g, 12.5mmol) was solved in 25 ml methanol and cooled on an ice bath. $SOCl_2$ (1.0ml, 13.0mmol) was slowly added under stirring. The ice bath was removed and the solution stirred overnight. The solvent was evaporated and the residue was submitted to flash chromatography on silica (CH_2Cl_2: MeOH, 9:1). The yield was 2.05g (93%) of a white solid.

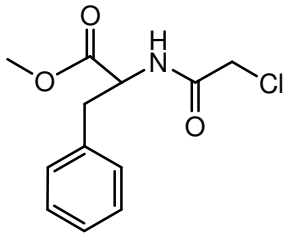
^{1}H NMR (300MHz, $CDCl_3$): δ 1.57 (s, 2H), 2.76-2.89 (q, 7.9 Hz, 1H), 2.88-3.11 (q, 5.1 Hz, 1H), 3.66 (s, 3H), 3.67-3.74 (m, 1H), 7.10-7.32 (m, 5H)

^{13}C NMR (300MHz, $CDCl_3$): δ 40.93, 51.81, 55.67, 126.69, 128.42, 129.12, 137.07, 175.25

MS (ES^+) (Mw=179): 180 ($M+H^+$)

(NMR-specter: Appendix 13)

(L)-Methyl 2-(2-chloroacetamido)-3-phenylpropanoate



15

(L)-methyl-2 amino-3-phenylpropanoate (0.50g, 2.8mmol) and K_2CO_3 (0.78g, 5.6mmol) was added to a solution of 25 ml dichloromethane and 10 ml acetonitrile. The solution was cooled on an ice bath and chloroacetyl chloride (231ml, 2.8mmol) was slowly added. The ice bath was removed and the solution was stirred at room temperature for 2 hours. The solution was filtered and evaporated. The residue was submitted to flash chromatography on silica (CH_2Cl_2: THF, 9:1). The yield was 0.40g (56%) of a colorless oil.

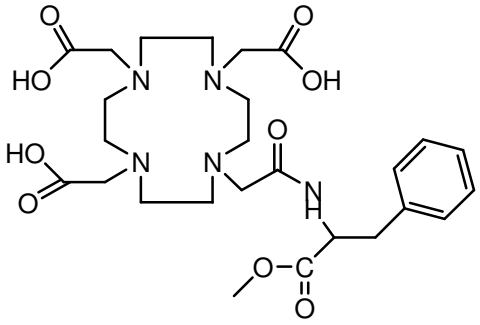
^{1}H NMR (300MHz, $CDCl_3$): δ 2.97-3.15 (m, 2H), 3.66 (s, 3H), 3.94 (s, 2H), 4.73-4.84 (m, 1H), 6.98-7.30 (m, 5H)

^{13}C NMR (300MHz, $CDCl_3$): δ 37.74, 42.33, 52.44, 53.35, 127.30, 128.65, 129.16, 135.28, 165.54, 171.20

MS (ES^+) (Mw=255): 256 ($M+H^+$)

(NMR-specter: Appendix 14)

1,4,7-Tris (carboxymethyl)- 10-[(L)-methyl 2-(2-acetamido)-3-phenylpropanoate]- 1,4,7,10-tetraazacyclododecan



16

(L)-methyl 2-(2-chloroacetamido)-3-phenylpropanoate (0.40g, 1.57mmol), DO3A (0.54g, 1.57mmol) and $NaHCO_3$ (0.66g, 7.86mmol) was dissolved in 10 ml water and 20 ml methanol. The solution was stirred at reflux for 16 hours and the solvent was then evaporated. The residue was submitted to flash chromatography on silica (MeOH: H_2O: NH_3 (25%), 9:1:0.1). The yield was 0.22g (24%) of a white solid.

^{1}H NMR (300MHz, D_2O): δ 1.30-3.90 (m, 29H), 4.45-4.60 (m, 1H), 7.10-7.33 (m, 5H)

^{13}C NMR (300MHz, D_2O): δ 37.58, 47.26, 51.88, 52.41, 55.89, 56.12, 57.99, 58.45, 126.17, 128.22, 128.65, 137.81, 172.45, 177.24, 178.81, 179.35, 179.43

MS (ES^+) (Mw=565): 566 ($M+H^+$)

(NMR-specter: Appendix 15)

6.3 Preparation of Gd(III) complexes

The ligand and $GdCl_3 \times 6H_2O$ were dissolved in water (10 ml) in equal amounts. The pH was adjusted to pH 7.0 by the addition of aqueous sodium hydroxide and the solution was stirred at 50°C for 2 hours. The pH was then adjusted to 9 by the addition of aqueous sodium hydroxide. The solution was filtered and the filtrate was evaporated.

Gd-DTPA was prepared by another method. $GdCl_3 \times 6H_2O$ (1.86g, 0.50mol) was solved in 20 ml water. The pH was adjusted to 11 by the addition of 5M NaOH. The solution was sentrifugated and the precipitate was washed with water until pH became neutral. The precipitate was transferred to a flask and 100 ml water and DTPA was added (1.98g, 0.50mol). The pH was adjusted to 7 by the addition of aqueous sodium hydroxide and the solution was refluxed until it became clear. At last the water was evaporated and the product was dried in vacuum.

6.4 Determination of albumin binding

Standard solutions of 1 mmol/ml Gd-DO3A-ADA, Gd-DO3A-MAD and Gd-DO3A-APP in phosphate buffer pH 7.4 were prepared. From each of those solutions two samples were made; One consisting of 1 ml standard solution and 1 ml phosphate buffer pH 7.4. The other consist of 1 ml standard solution and 1 ml 8% BSA. The samples were heavy stirred and put on a water bath at 37°C for 1 hour. Before the samples were filtered, a small amount of the phosphate buffer solution was taken out as a control to see if some of the substances were lost during the filtration. The samples were then transferred to Centricon filters (Centrifugal filter devices 10 000 Mw cut off) and sentrifugated at 37°C for 1 hour (4000 rpm). After sentrifugation the concentration of unbound substance in filtrate was determined on HPLC. The HPLC method used was an isocratic eluation consisting of acetonitrile in aqueous H_3PO_4, pH 2.1. The flow rate was 0.6 ml/min. The albumin binding was measured three times for each substance.

6.5 Determination of T_1- relaxivity

The substances Gd-DO3A-ADA, Gd-DO3A-MAD and Gd-DO3A-APP were prepared at concentrations from 1.25mM to 20mM in phosphate buffer- and BSA-solutions. The relaxation time was measured at 40°C. BSA solutions were 4% BSA in phosphate buffer pH 7.4 (in one measurement a 10% BSA solution was used to make a 1:1 ratio between albumin and the complex). Relaxation time of DTPA-Gd was measured and used as a control. The T_1- relaxivity of the complexes was calculated from the following equation:

$$r_1 = \frac{R_1^{obs} - R_1^{m}}{[Gd(\mathrm{III})]}$$

where R_1^{obs} is the observed relaxation rate which in time is $1/T_1^{obs}$ and R_1^{m} is the relaxation rate of the matrix which in time is $1/T_1^{m}$. [Gd(III)] is the concentration of Gd(III) in mM.

6.6 Displacement

In the first strategy a 2.5mM solution of Gd-DO3A-MAP in BSA was prepared. 16ml of this solution was equally divided into four test tubes. The first was added an amount of oxazepam to give a concentration of 5mM. The second was added an amount of warfarin to give a concentration of 5mM, and the third was added an amount of caprylic acid to give a concentration of 5mM. The last was used as a control. The relaxation was measured for each of those test tubes.

In the second strategy a 5mM solution of Gd-DO3A-MAP and an 8% solution of BSA was prepared, both in phosphate buffer pH 7.4. Four test tubes were added 2ml of the BSA solution and the substances warfarin, oxazepam and caprylic acid was each added to one test tube to give a concentration at 10mM. The albumin solutions with the added substances were stirred and 2ml of the Gd-DO3A-MAP were added to each tube. The tubes were stirred again and the relaxivity was measured. If any of the added substances would displace Gd-DO3A-MAP from BSA, a decrease in the relaxivity was expected.

7 REFERENCES

[1] Bloch, F. Phys. Rev. (1946), 70, 460

[2] Purcell, E.M; Torrey, H.C; Pound, R.V. Phys. Rev. (1946), 69, 37

[3] The Nobel Foundations internet page http://nobelprize.org/physics/laureates/1952/

[4] The US National Academy of Sciences internet page. http://www.beyonddiscovery.org/content/view.txt.asp?a=129

[5] Smith, H.J, Tidsskr Nor Laegeforen. (2003), 123(23), 3352

[6] Lauterbur, P.C; Mendoza-Diaz, M.H; Rudin, A.M. *Frontiers of Biological Energetics*, eds. P.L. Dutton, L.S.Leigh, A.Scarpa, Academic, New York, (1978), 752

[7] Shellock, F.G; Kanal, E,J Magn Reson Imaging. (1999), 10(3), 477

[8] Caravan, P; Ellison, J.J; McMurry, TJ; Lauffer, R.B. Chem Rev. (1999), 99(9), 2293

[9] Cavagna, F.M; Maggioni, F; Castelli, P.M.; Dapra, M; Imperatori, L.G; Lorusso, V; Jenkins, B.G. Investigative Radiology (1997), 32(12), 780

[10] Tóth, E; Kiraly, R; Platzek, J; Raduechel, B; Bruecher, E. Inorg. Chim. Acta, (1996), 249(2), 191

[11] GE Healthcare internet page http://www.amershamhealth.com/medcyclopaedia/Volume%20I/MR%20contrast%20medium.asp

[12] Aime, S; Fasano, M; Terreno, E. Chemical Society Reviews (1998), 27(1), 19

[13] Burai, L; Scopelliti, R; Toth, E. Chemical Communications (2002), 2(20), 2366

[14] Moats, R.A; Fraser, S.E; Meade, T,Angewandte Chemie, Int. Ed. Eng (1997), 36(7), 726

[15] Fossheim, S.L; Il'yasov, K.A; Hennig, J; Bjornerud, A, Academic radiology (2000), 7(12), 1107

[16] Caravan, P; Comuzzi, C; Crooks, W; McMurry, T.J; Choppin, G.R; Woulfe, S.R. Inor. Chem. (2001), 40(9), 2170

[17] Caravan, P; Cloutier, N.J; Greenfield, M.T; McDermid, S.A.; Dunham, S.U; Bulte, J.W.M.; Amedio, J.C.Jr; Looby, R.J; Supkowski, R.M.; Horrocks, W.DeW.Jr; McMurry, T.J; Lauffer, R.B. Journal of the American Chemical Society (2002), 124(12), 3152

[18] Bhattacharya, A.A; Grune, T; Curry, S. Journal of Molecular Biology, (2000), 303(5), 721

[19] Felleskatalogen 2004, Felleskatalogen AS, Oslo, Norway

[20] Schultze, L; Bulls, A.R. WO 96/28433/1996

[21] Greene, T.W; Wuts, P.G.M, Protective groups in organic synthesis, 2nd edition.,John Wileys and Sons,Inc, New York, 1991.

[22] Sakaguchi, J; Higashi, T; Azuma, T; Suzuki, T; Iwasaki, N; Kondo, N; Nagata, O; Kato, H; Hanaoka, M., Chemical & Pharmaceutical Bulletin (2001), 49(6), 788

[23] Aime, S; Gianolio, E; Terreno, E; Giovenzana, G.B; Pagliarin, R; Sisti, M; Palmisano, G; Botta, M; Lowe, M.P; Parker, D. JBIC, Journal of Biological Inorganic Chemistry (2000), 5(4), 488

[24] Tweedle, M.F; Gaughan, G.T; Hagan, J.J., EP 292689/1988

[25] Brenner, M; Huber, W, Helvetica Chimica Acta (1953), 36, 1109

[26] Chenault, H.K; Dahmer, J; Whitesides, G, M.,Journal of the American Chemical Society (1989), 111(16), 6354

[27] Fehske, K.J.; Mueller, W.E.; Wollert, U. Biochemical Pharmacology (1981), 30(7), 687

[28] Nakamoto, I; Morimoto, K. Environmental Health and Preventive Medicine (2004), 9(2), 63

[29] Trynda-Lemiesz, L. Bioorganic & Medicinal Chemistry (2004), 12(12), 3269

[30] Sudlow,G; Birkett,D.J; Wade,D.N. *Mol. Pharmacol.*, (1975), 11(6), 824

[31] Sudlow,G; Birkett,D.J; Wade,D.N. *Mol. Pharmacol.*, (1976), 12(6), 1052

[32] Sugio,S; Kashima, A; Mochizuki, S; Noda, M; and Kobayashi, K. Protein Engineering, (1999), 12(6), 439

[33] Henrotte, V; Laurent, S; Gabelica, V; Vander Elst, L; Depauw, E; Muller, R. N. Rapid Communications in Mass Spectrometry (2004), 18(17), 1919

[34] McMurry T.J; Parmelee D.J; Sajiki H; Scott D.M; Ouellet H.S; Walovitch R.C; Tyeklar Z; Dumas S; Bernard P; Nadler S; Midelfort K; Greenfield M; Troughton J; Lauffer R.B; J Med Chem. (2002), 45(16), 3465

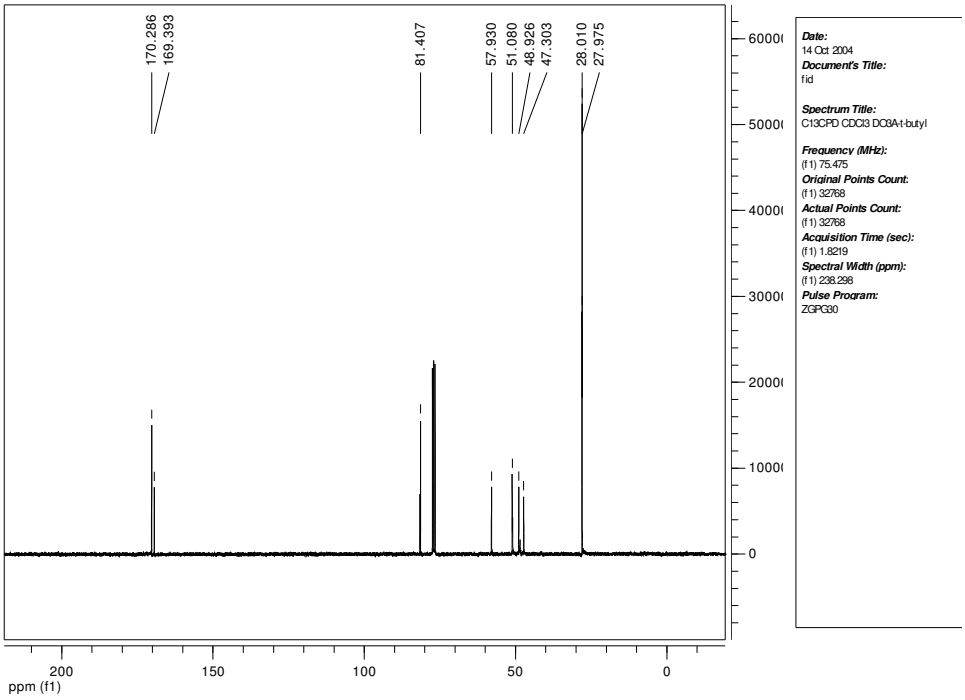
[35] Wiegers C.B; Welch M.J; Sharp T.L; Brown J.J; Perman W.H; Sun Y; Motekaitis R.J; Martell A.E; Magn Reson Imaging. (1992), 10(6), 903

[36] Bos, O.J.M; Remijn, J.P.M; Fischer, M.J.E; Wilting, J; Janssen, L.H.M. Biochemical Pharmacology (1988), 37(20), 3905

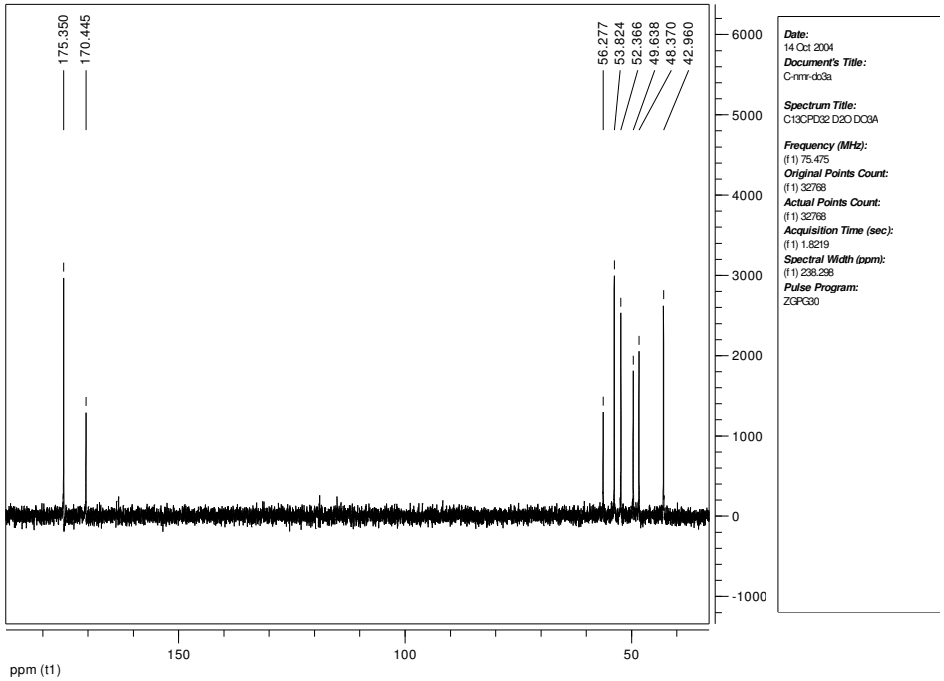
8 APPENDICES

Appendix 1

1.1 1,4,7-Tris(carboxymethyl-tert-butylester)-1,4,7,10-tetraazacyclodecane (DO3A-tert-butyl)

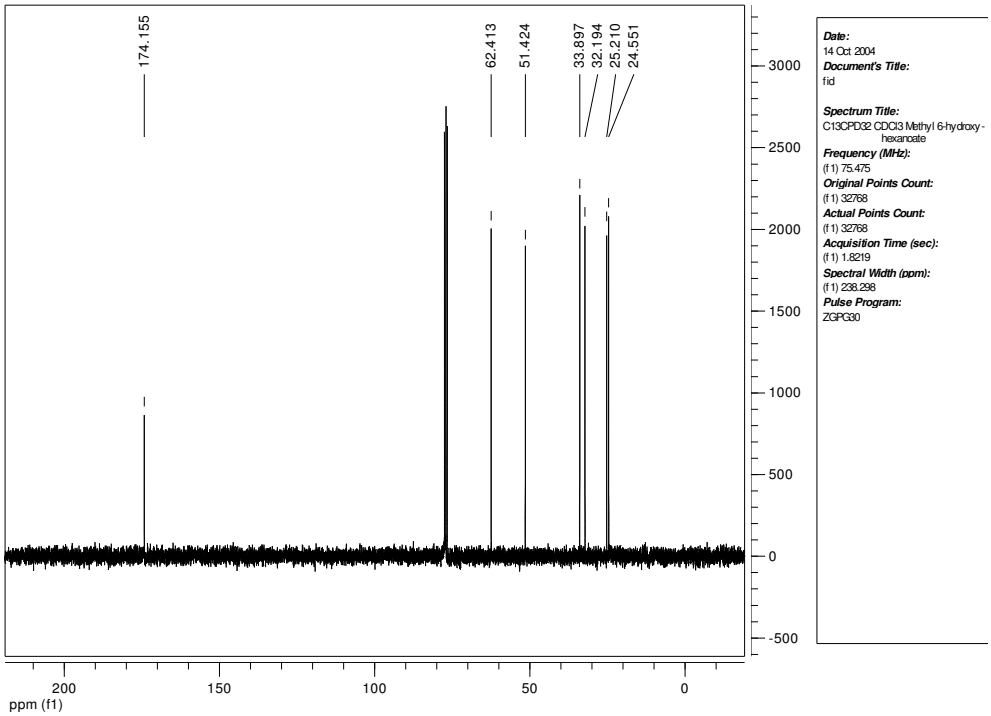


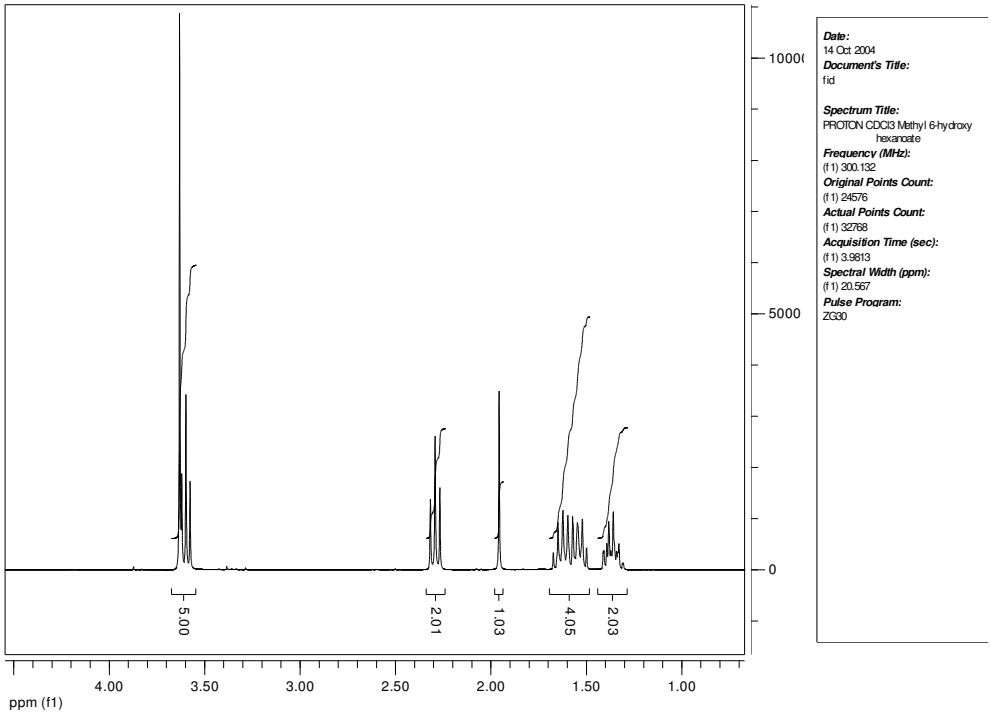
1.2 1,4,7-Tris(carboxymethyl)-1,4,7,10-tetraazacyclodecane (DO3A)



Appendix 2

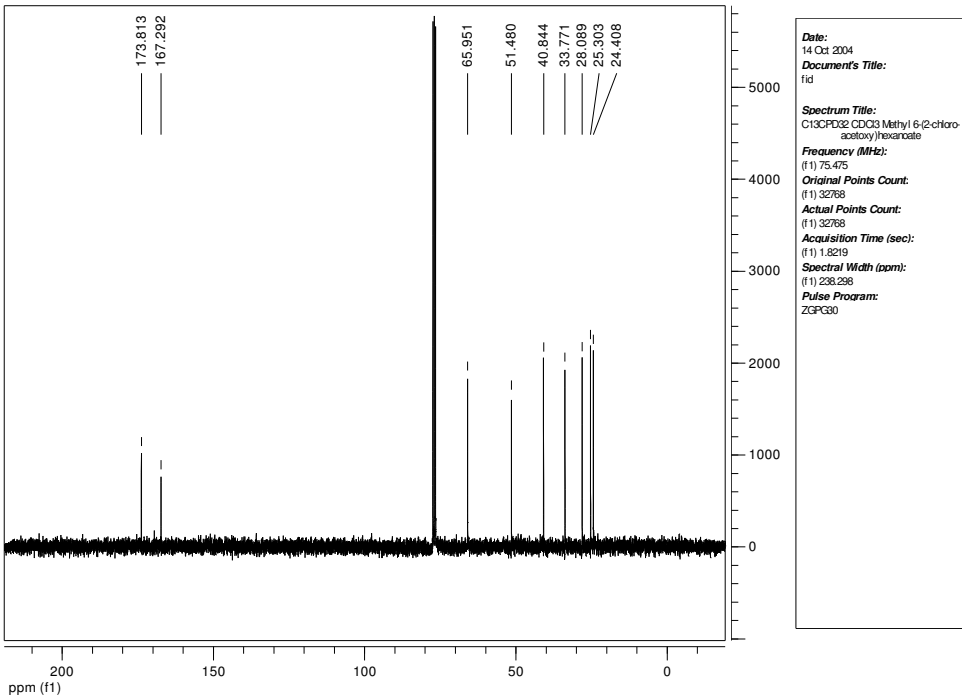
Methyl-6-hydroxyhexanoate

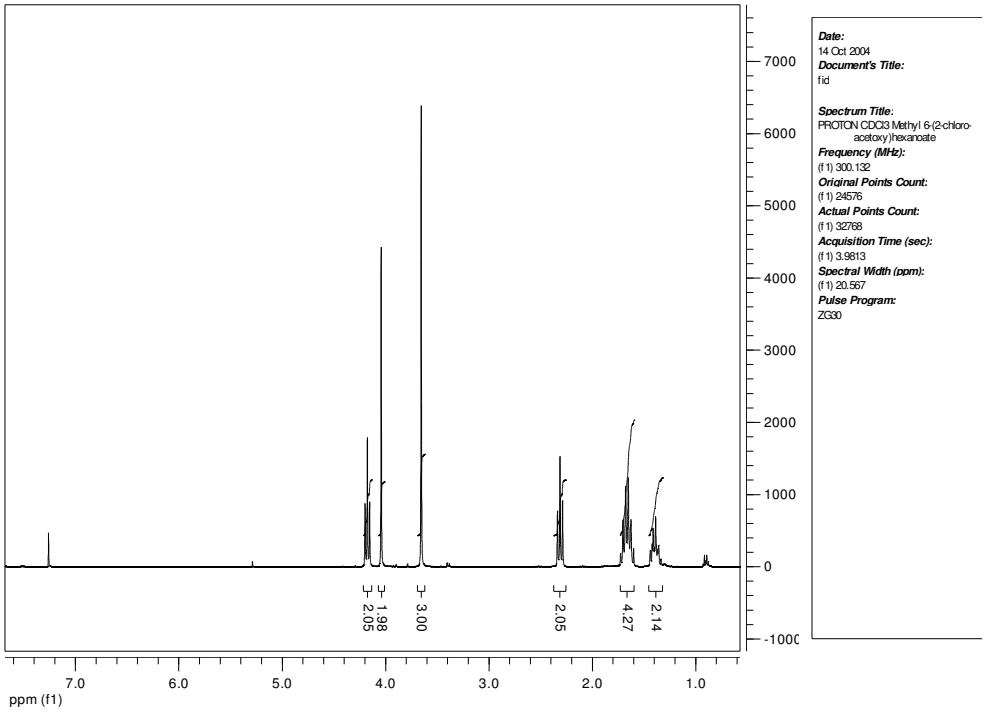




Appendix 3

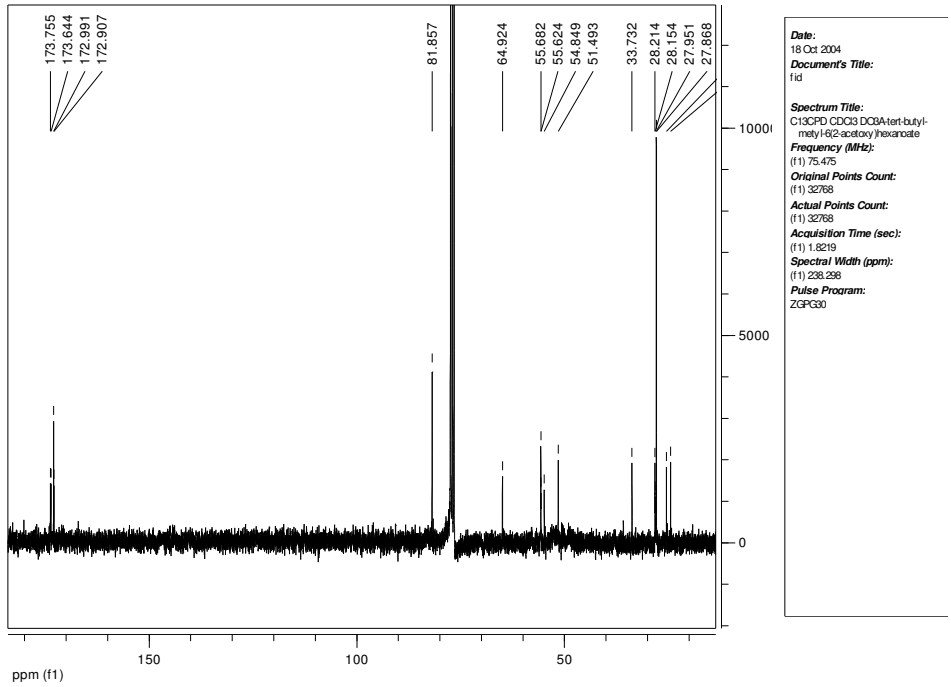
Methyl 6-(2-chloroacetoxy)hexanoate

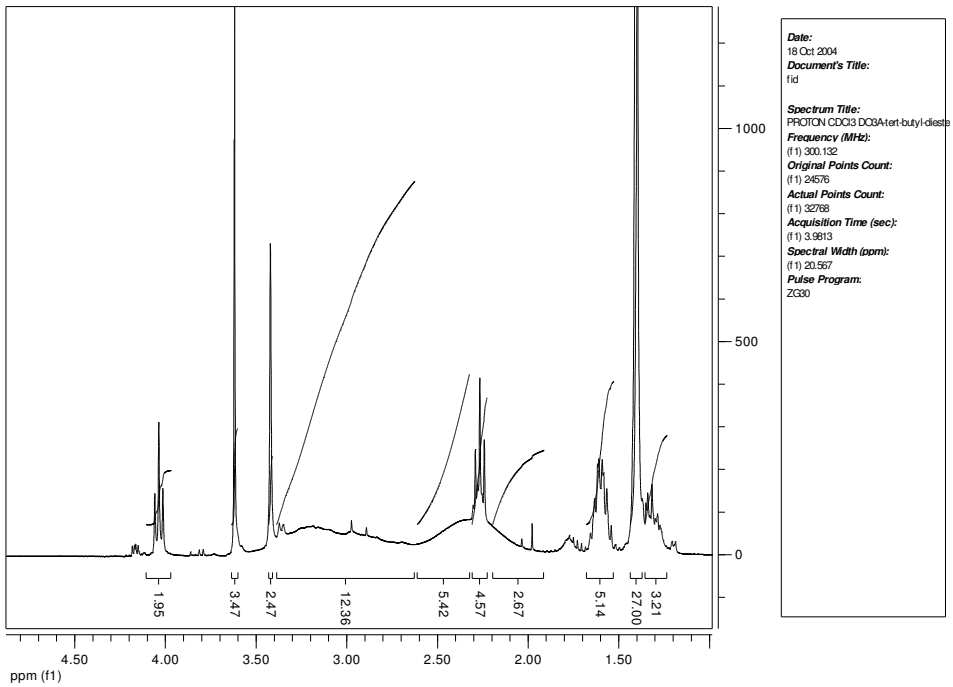




Appendix 4

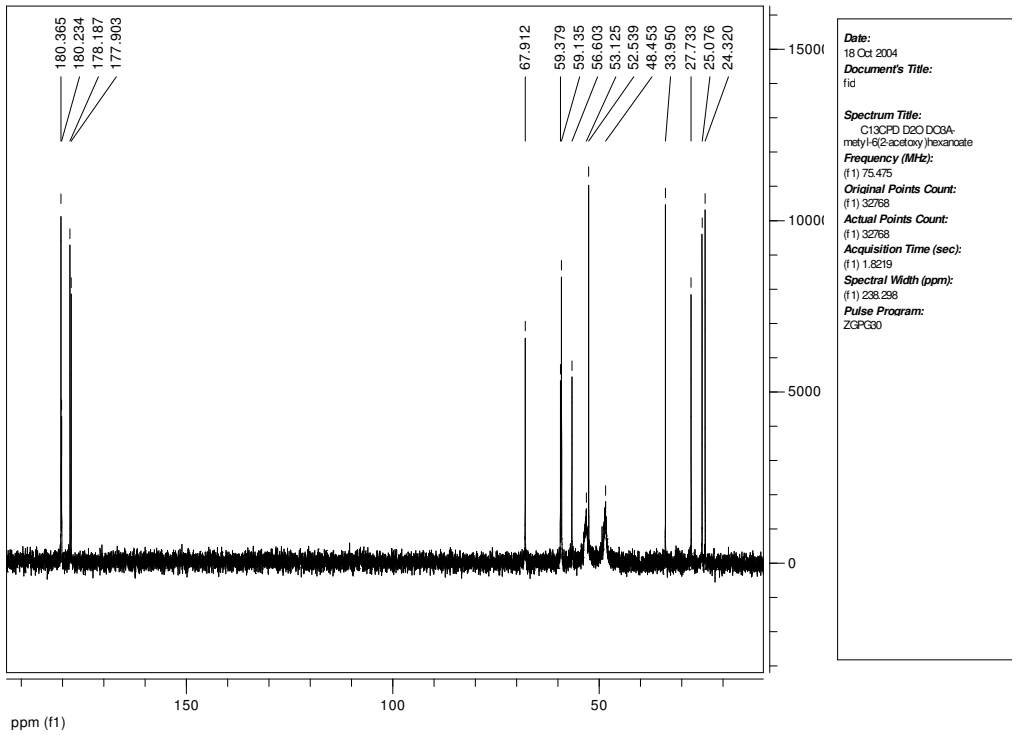
1,4,7-Tris (tert-butylcarboxymethyl)- 10-[metyl-6(2-acetoxy)hexanoate]- 1,4,7,10-tetraazacyclododecan

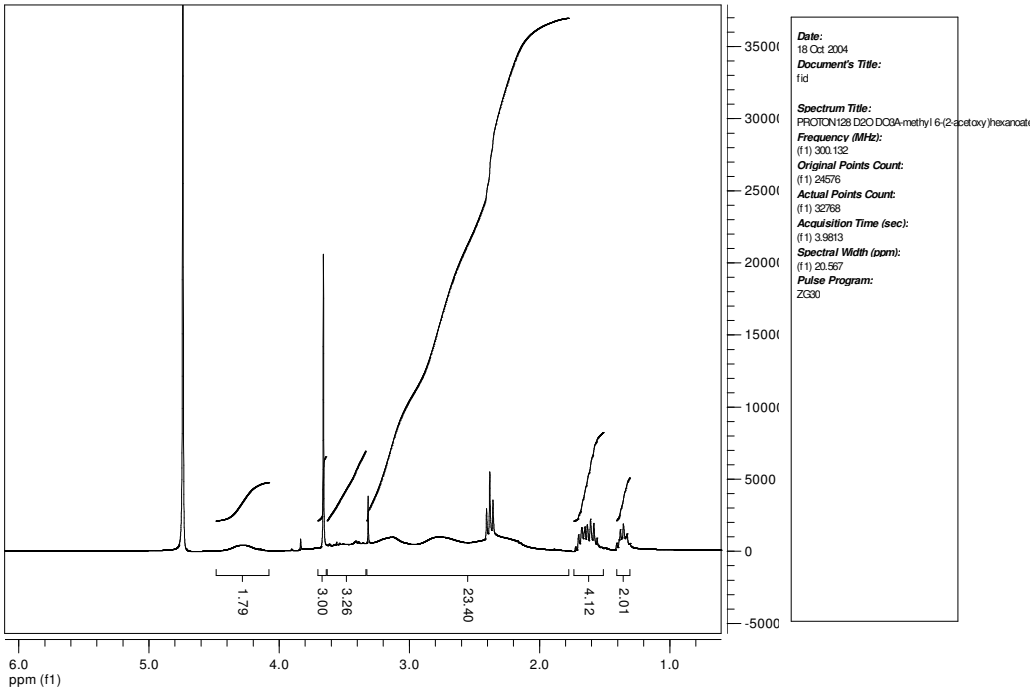




Appendix 5

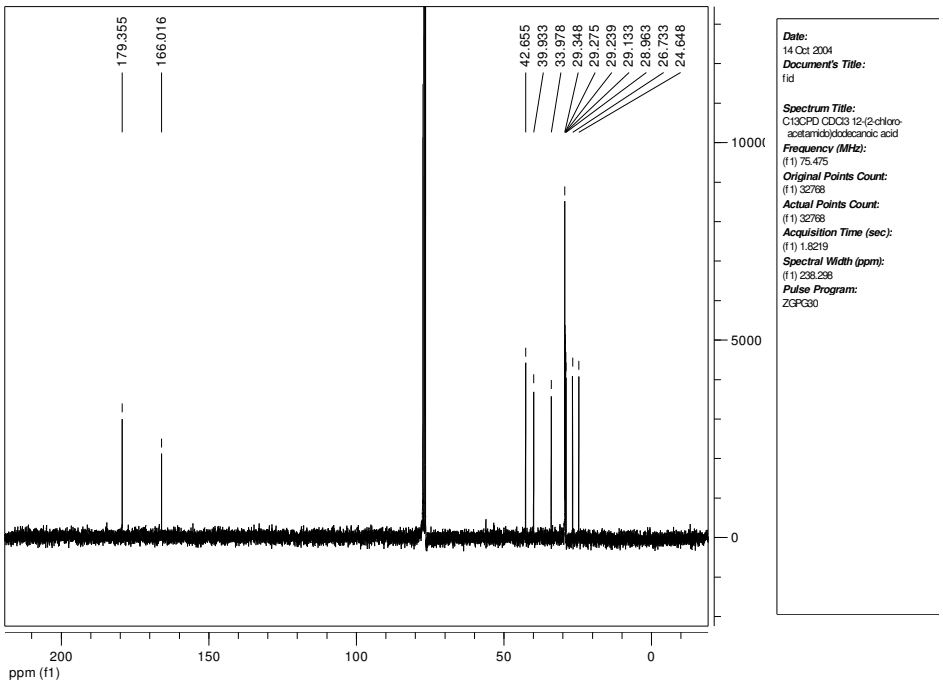
1,4,7-Tris (carboxymethyl)- 10-[metyl-6(2-acetoxy)hexanoate]- 1,4,7,10-tetraaza-cyclododecan

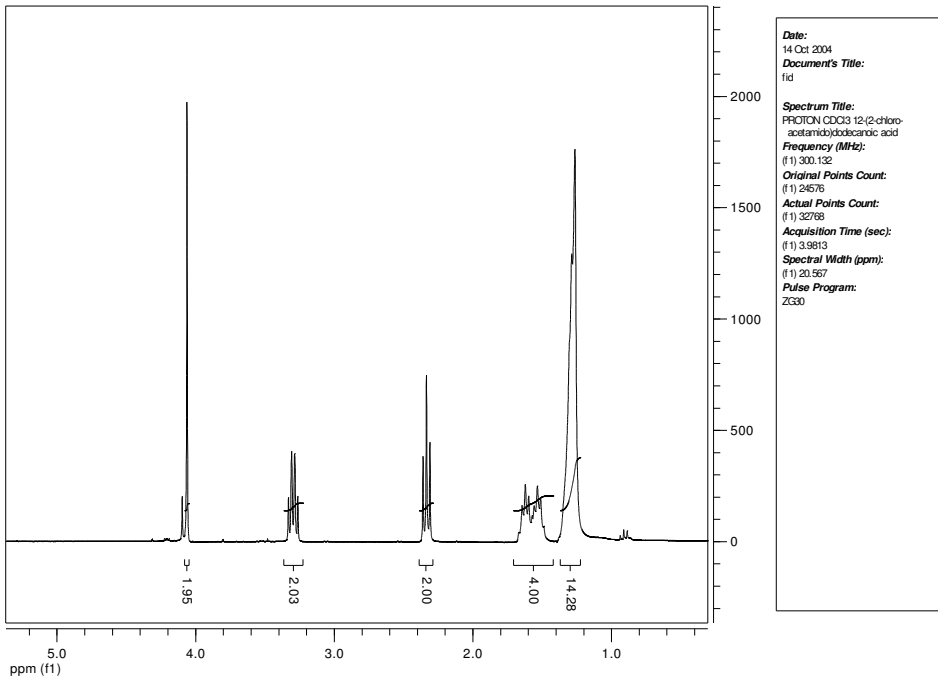




Appendix 6

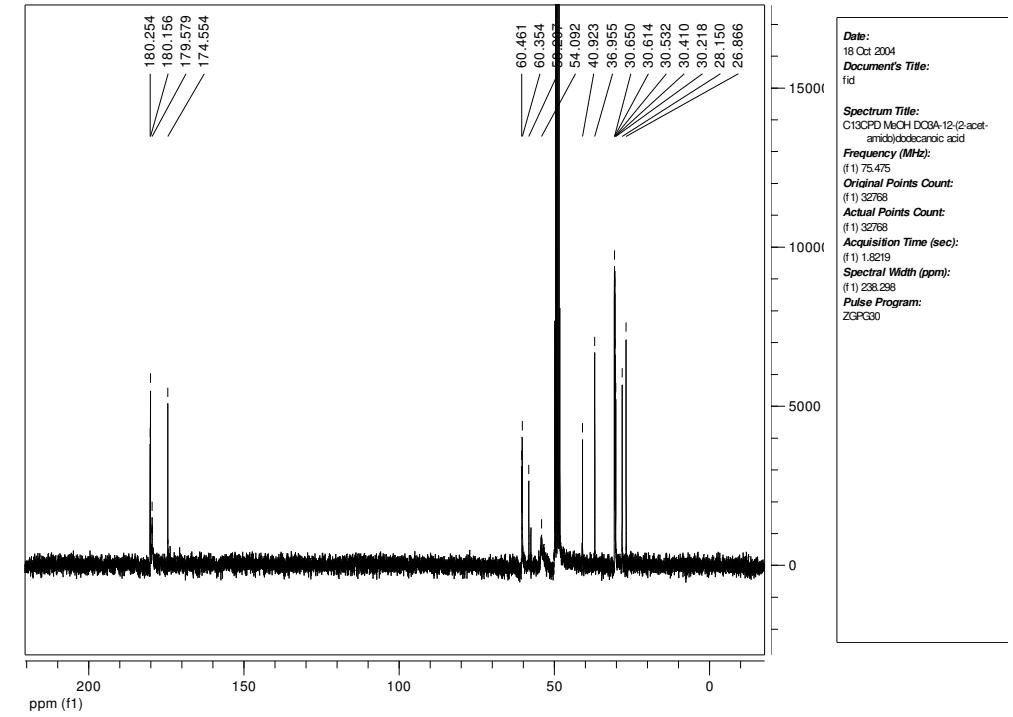
12-(2-Chloroacetamido)dodecanoic acid

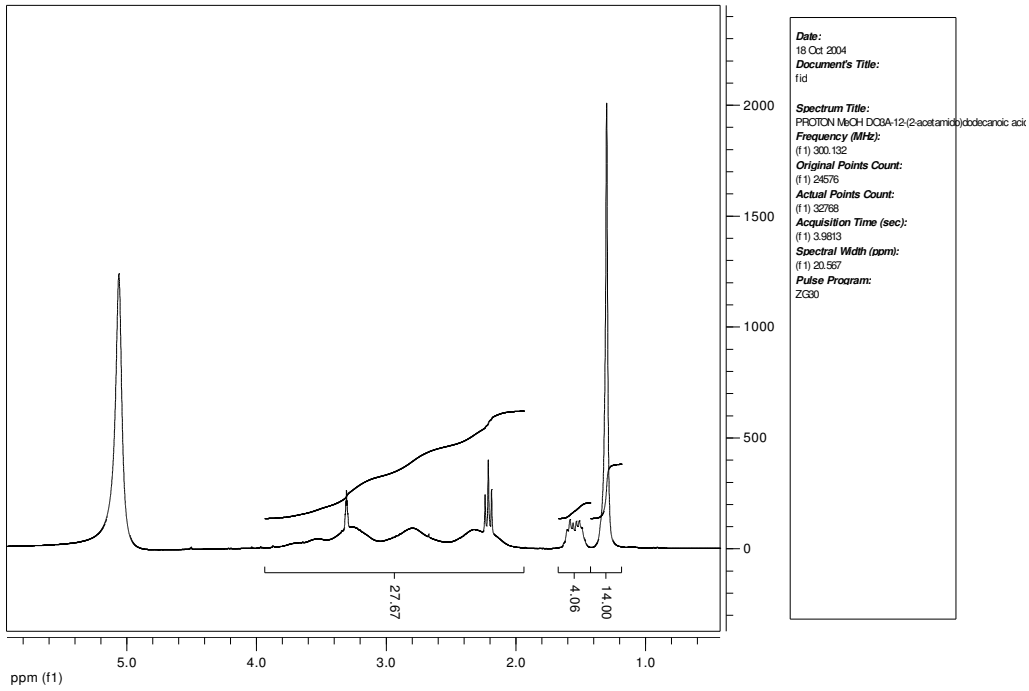




Appendix 7

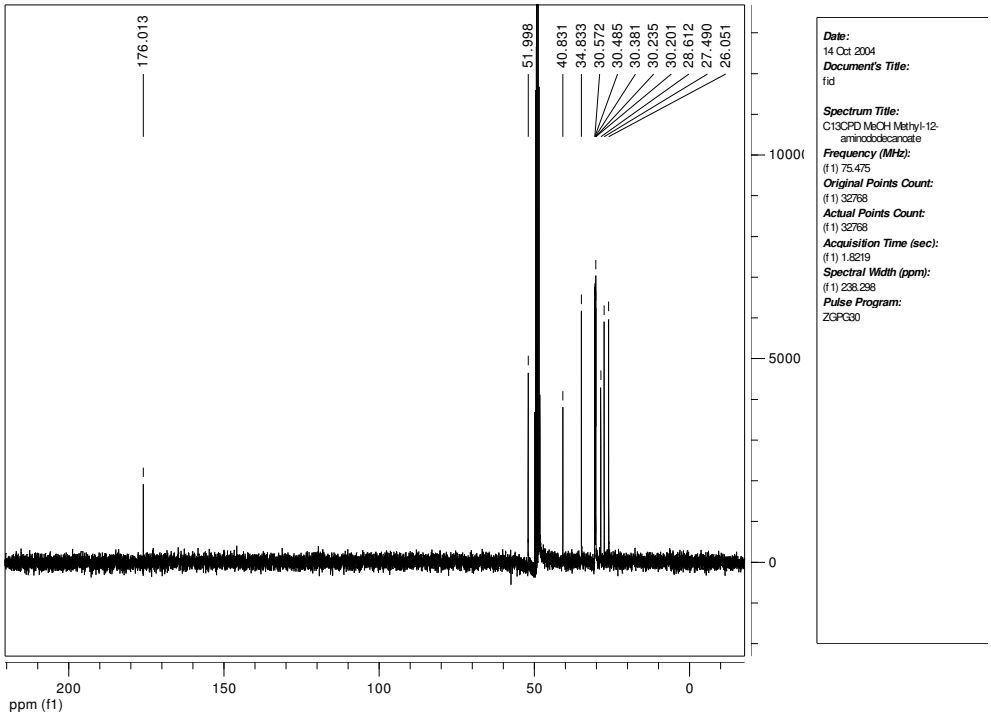
1,4,7-Tris (carboxymethyl)- 10-[12-(2-acetamido)dodecanoic acid]- 1,4,7,10-tetraaza-cyclododecan

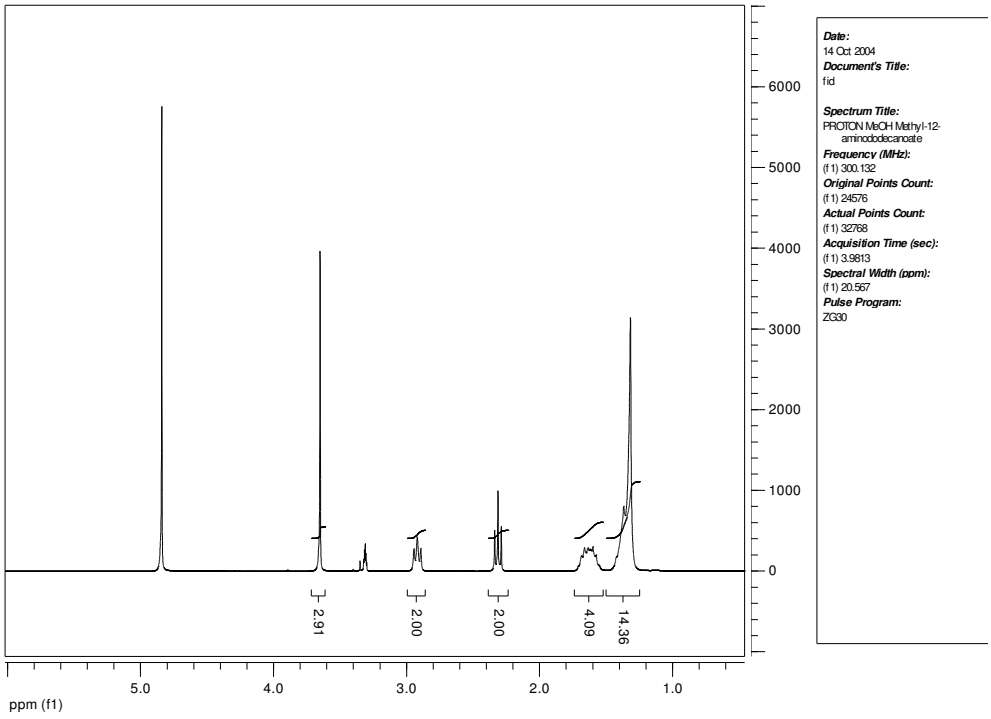




Appendix 8

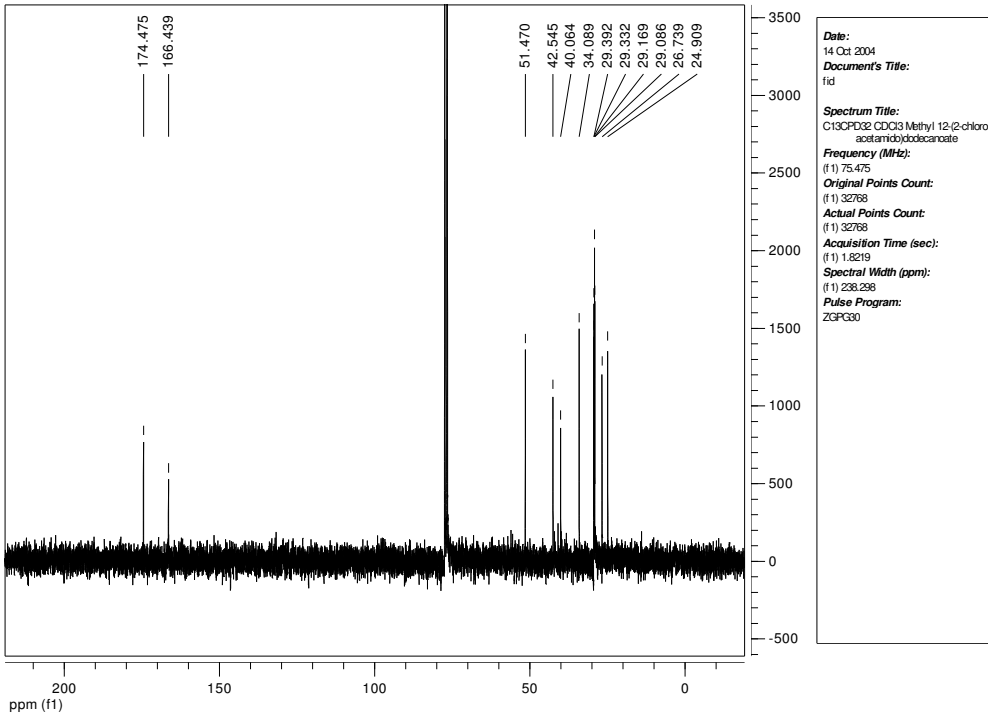
Methyl-12-aminododecanoate

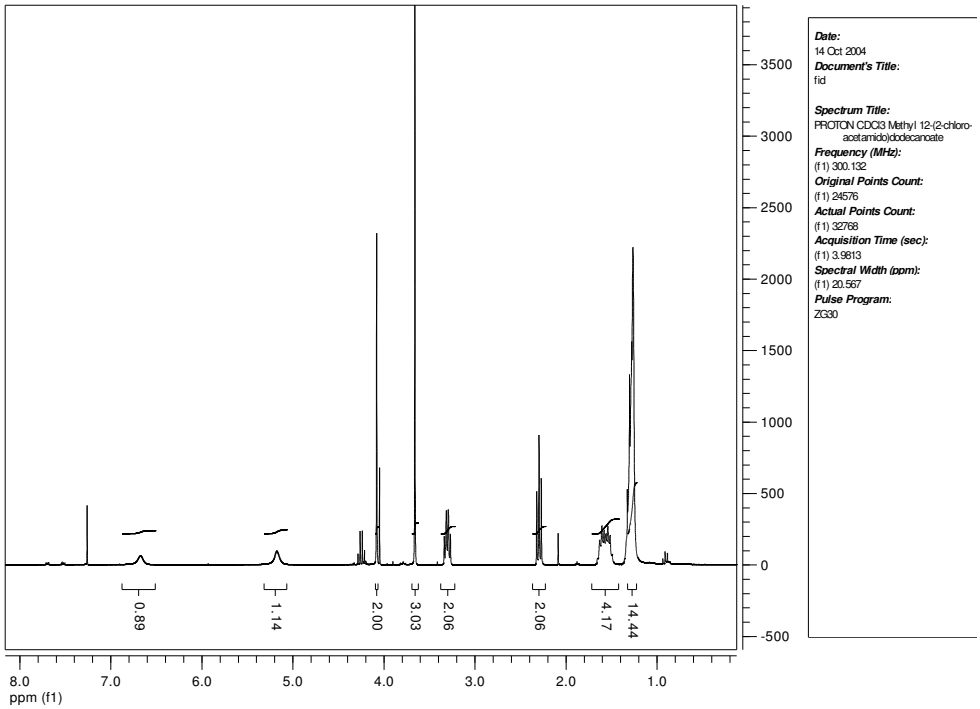




Appendix 9

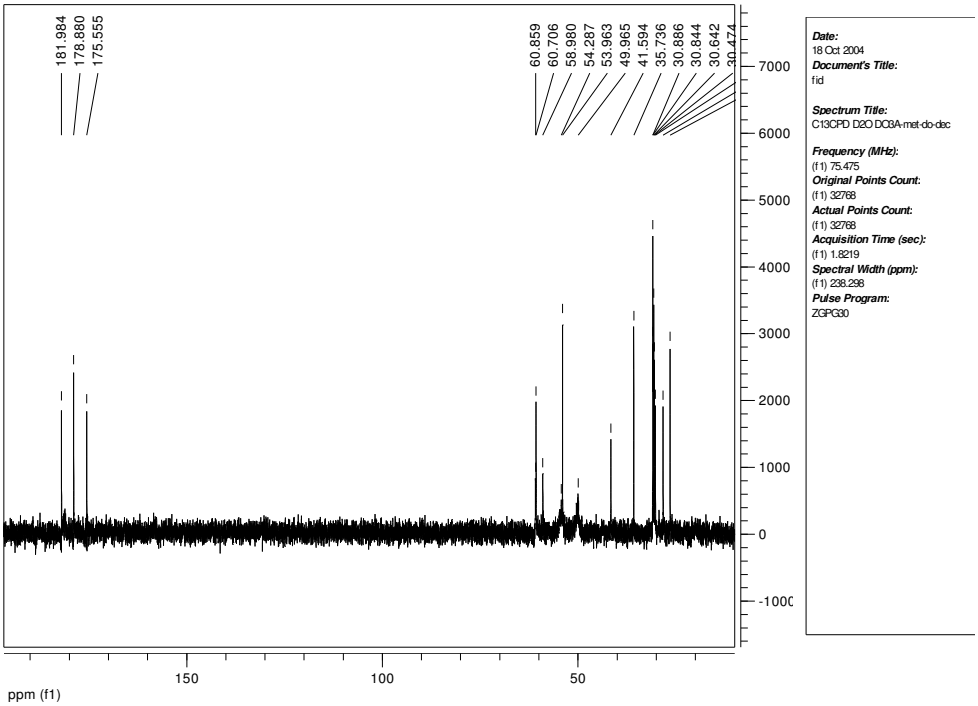
Methyl 12-(2-chloroacetamido)dodecanoate

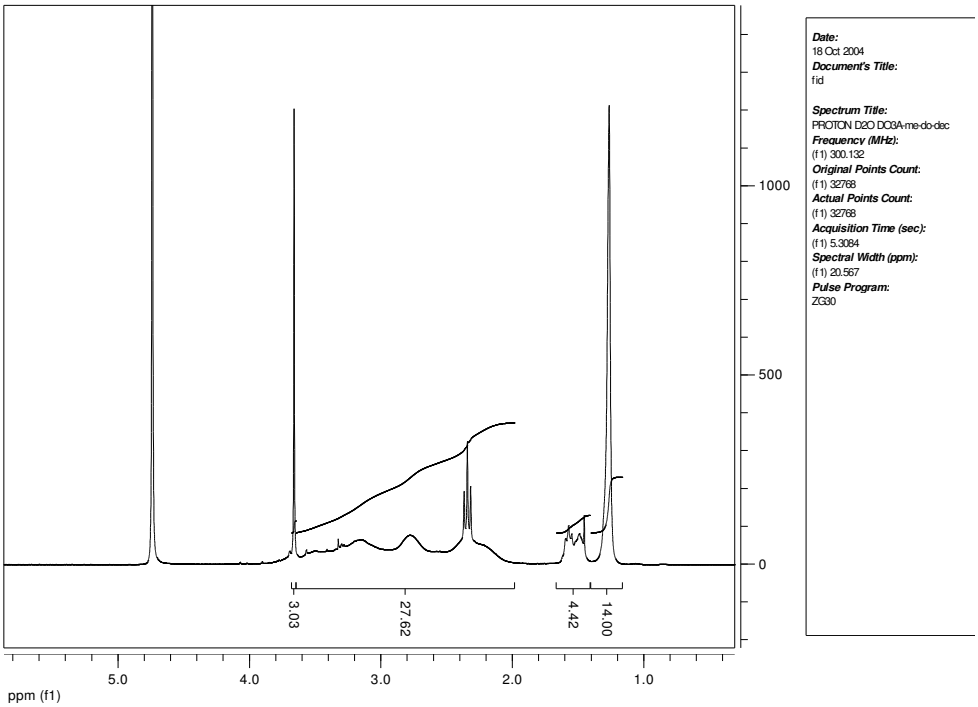




Appendix 10

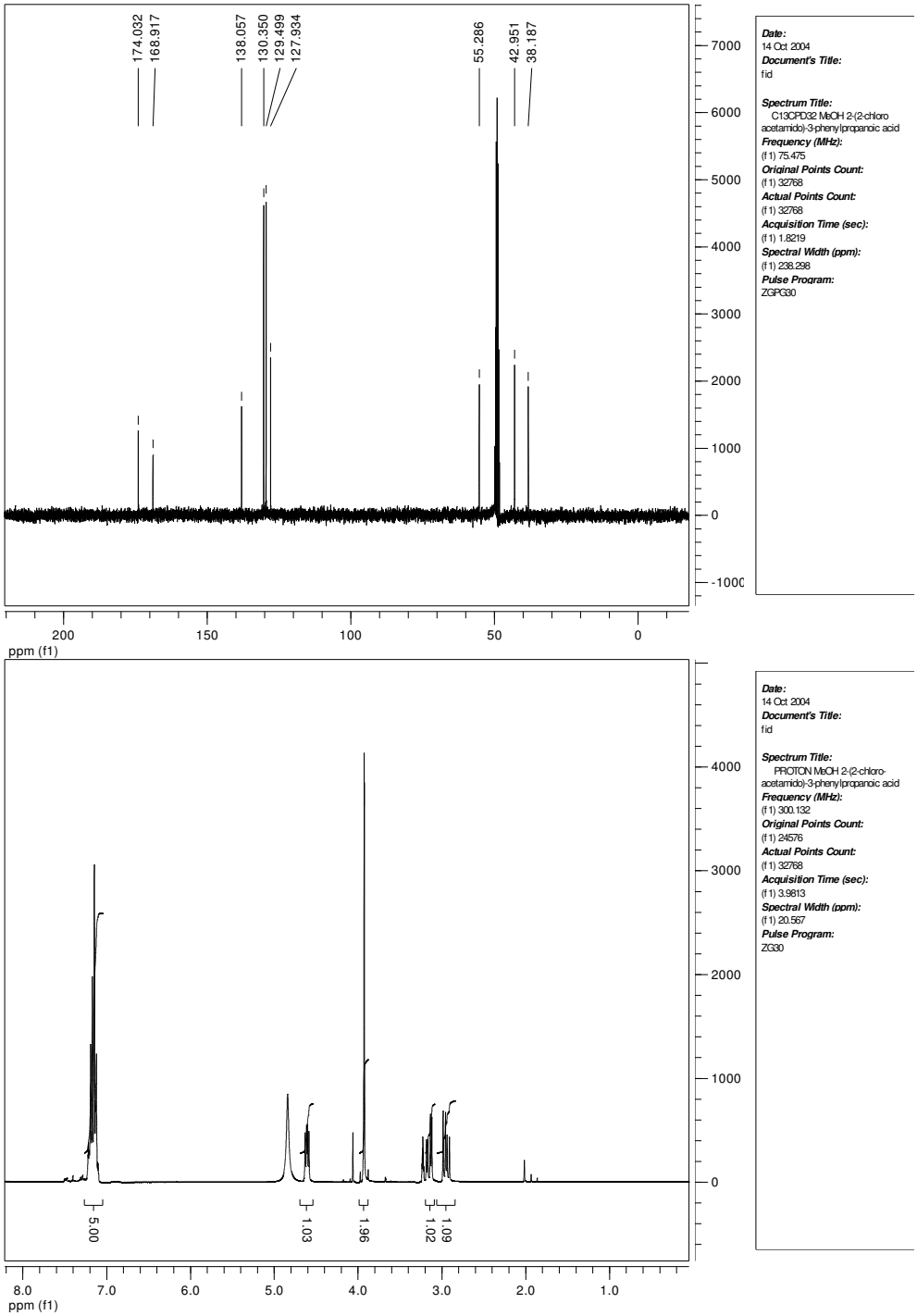
1,4,7-Tris (carboxymethyl)- 10-[methyl 12-(2-acetamido)dodecanoate] - 1,4,7,10-tetraazacyclododecan





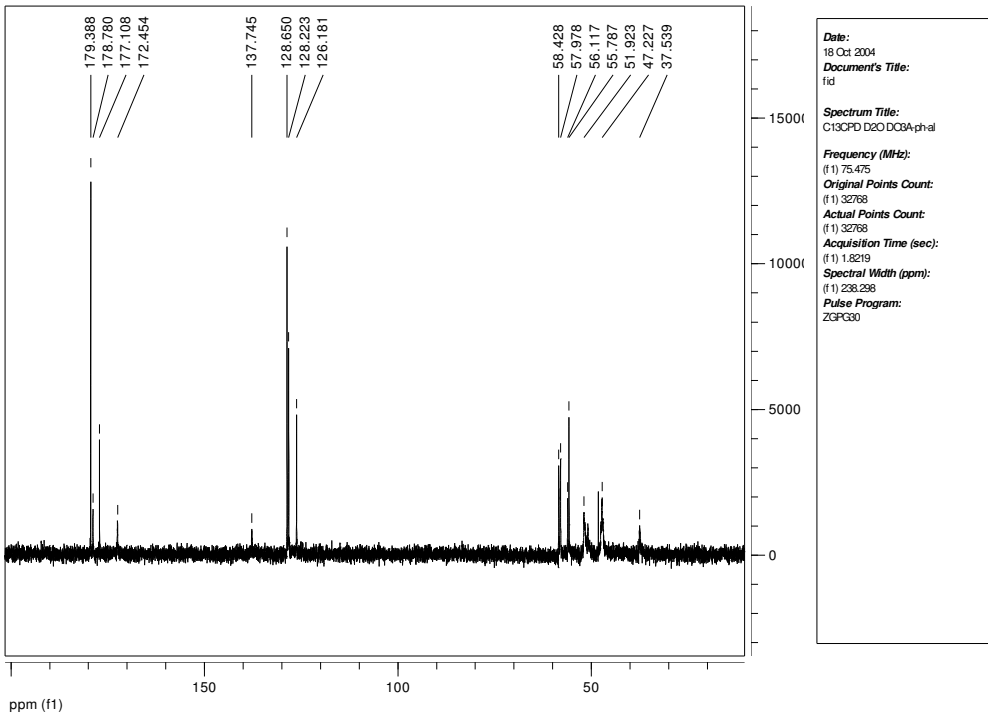
Appendix 11

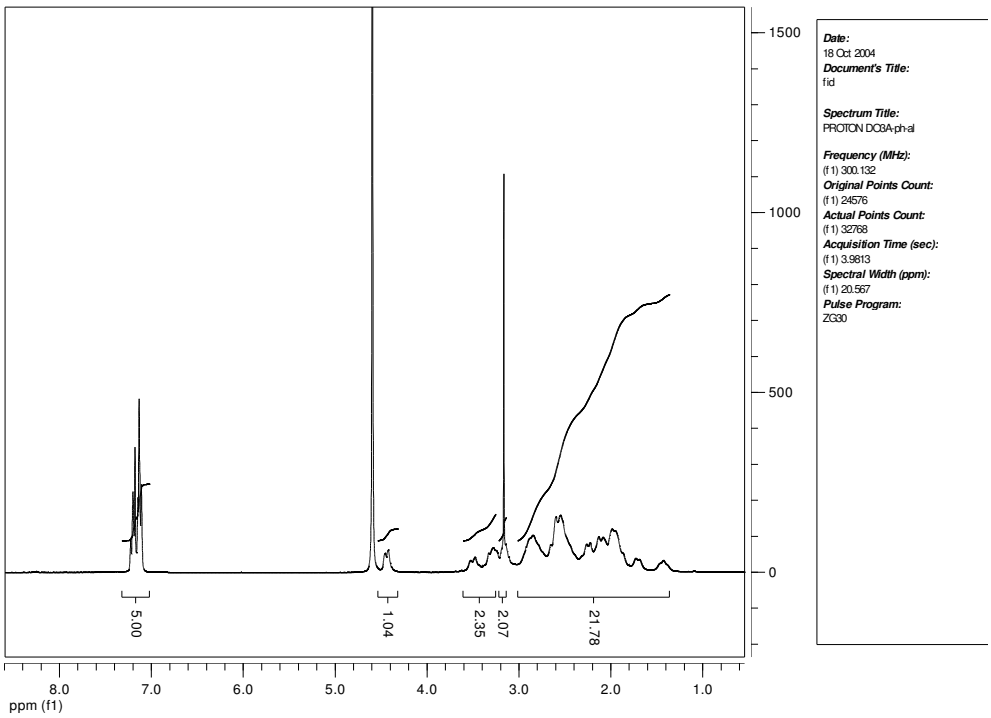
(L)-2-(2-Chloroacetamido)-3-phenylpropanoic acid



Appendix 12

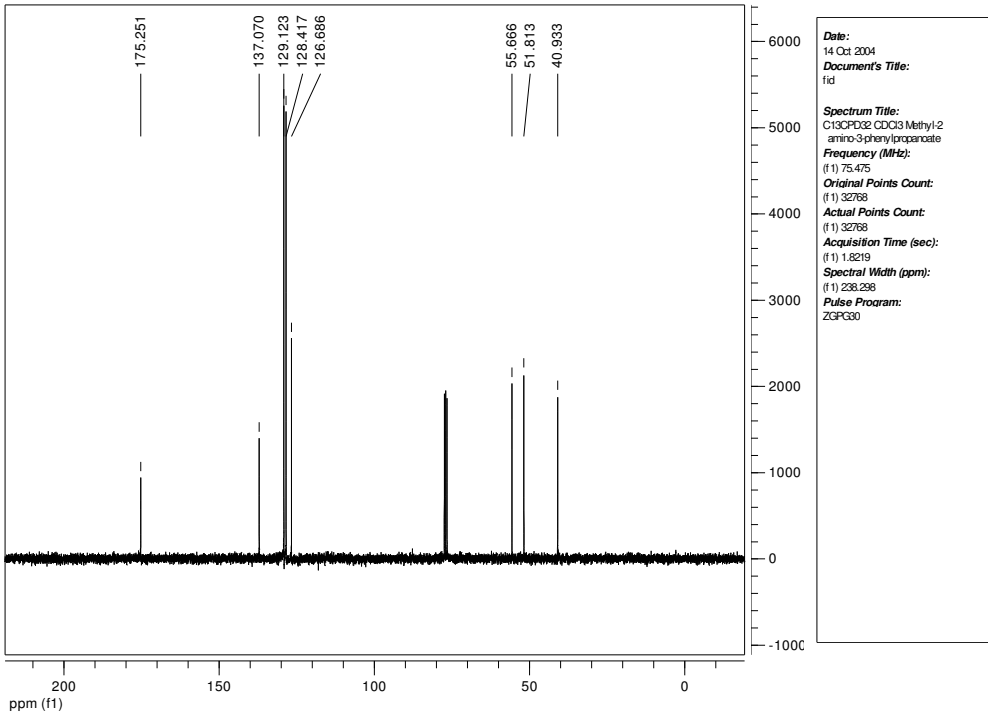
1,4,7-Tris (carboxymethyl)- 10-[(L)-2-(2-acetamido)-3-phenylpropanoic acid]- 1,4,7,10-tetraazacyclododecan

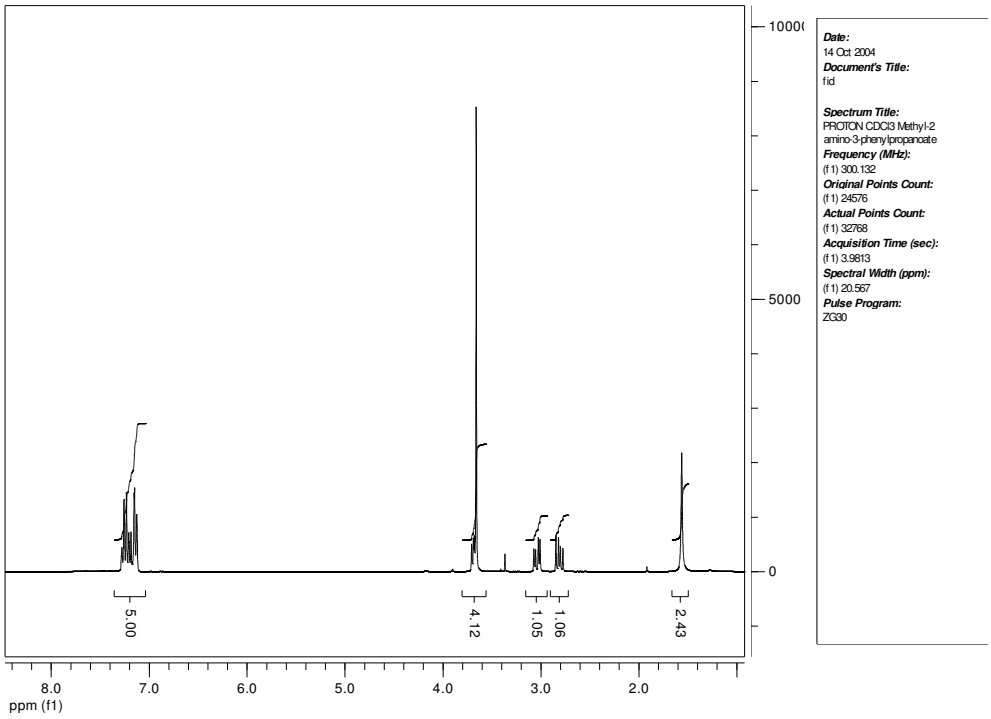




Appendix 13

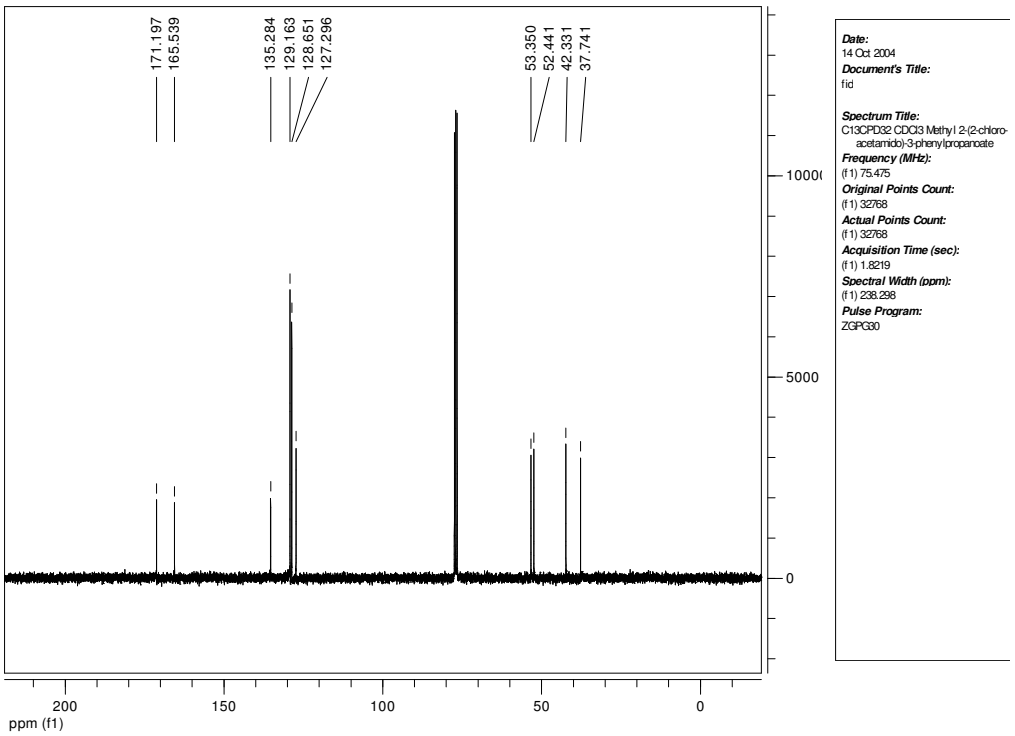
(L)-Methyl-2-amino-3-phenylpropanoate

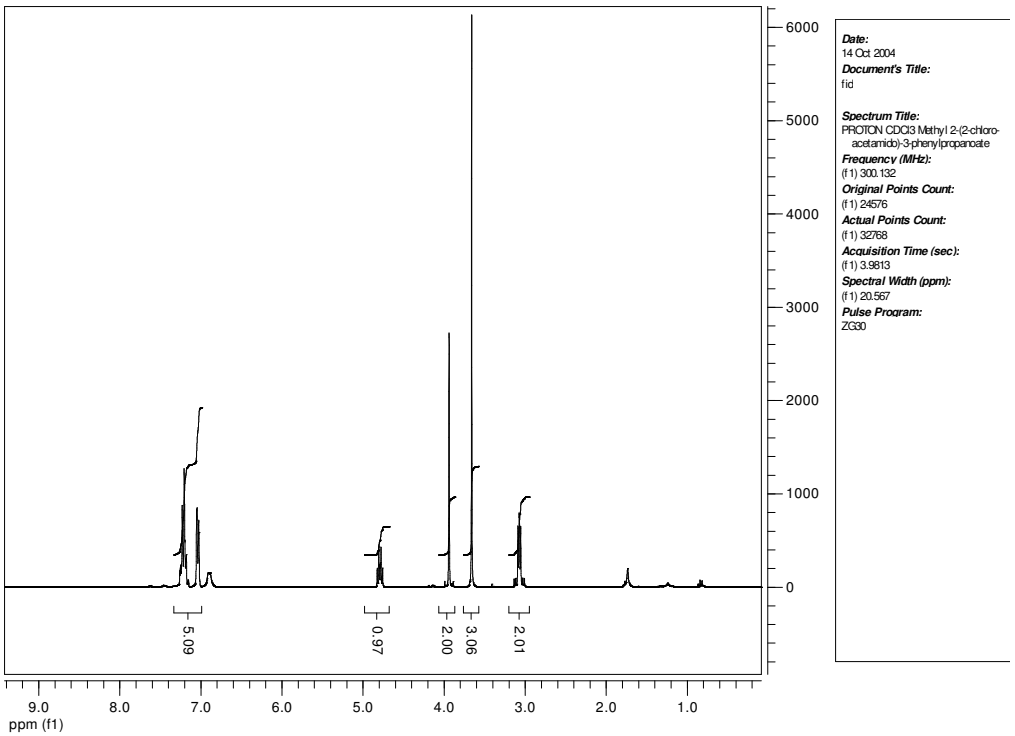




Appendix 14

(L)-Methyl 2-(2-chloroacetamido)-3-phenylpropanoate





Appendix 15

1,4,7-Tris (carboxymethyl)- 10-[(L)-methyl 2-(2-acetamido)-3-phenylpropanoate]- 1,4,7,10-tetraazacyclododecan